THE ACTIVATION AND ACTION OF T LYMPHOCYTES

by

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STATEMENT

The RNA hybridization experiments described in chapter 3 and 4 were performed in conjunction with Rosemarie Johnson and Dr. Ian Young of the Medical Molecular Biochemistry Unit, JCSMR. Andrew Hapel of the Dept. Medicine and Clinical Science, JCSMR, tested the effect of cyclosporine on lymphokine release from tumour cell lines described in chapter 4. The experiments described in chapter 5 were carried out in conjunction with Dr. Michael Agostino and Jane Dixon (then of the Transplantation Biology Unit, JCSMR) and Karen Sellin and Kevin Lafferty (The Barbara Davis Centre for Childhood Diabetes, Denver). The modelling and computer simulation of lymphokine release described in chapter 7 was done in collaboration with David McKinnon of the Dept. of Physiology, JCSMR. With these exceptions the experiments reported in this thesis were performed by myself.

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ABSTRACT

The work presented in this thesis examines the *in vitro* characteristics, and the *in vivo* role of antigen-triggered lymphokine release from activated T cells.

Analysis of cell dose response curves revealed that release of two lymphokines, interleukin 2 (IL2) and interleukin 3 (IL3) can be triggered solely by antigen stimulation. Thus, either lymphokine can be used to study antigen exposure of the T cell.

A closer examination of cell dose curves for IL3 release revealed the cellular requirements for T cell stimulation by the 'polyclonal' T cell mitogens, concanavalin A (Con A) and $NaIO_4$. These agents do not signal T cells following direct contact but must be 'presented' on the surface of another cell before the antigen-equivalent signal is triggered.

A steady state binding model of T cell-target cell interaction was derived and could successfully describe the observed cell dose response curves for lymphokine release if the assumption was made that the reaction, triggering of lymphokine release, is an all or none event.

An analysis of the site of action of cyclosporine (CsA)

revealed a specific inhibitory action on transmission of the antigen signal. This drug was used *in vivo* to show the importance of lymphokine release in T cell-dependent reactions.

The possible application of these results to the development of quantitative methods of assaying activated T lymphocytes as well as the more general implications of how the results might relate to the development of immune response heterogeneity are discussed in the final chapter.

ABBREVIATIONS

32D	32D-cl IL3 dependent cell line
B6	C57Bl/6J
C'	Complement
CBA	CBA/H
CMI	Cell mediated immunity
Con A	Concanavalin A
c.p.m.	Counts per minute
CS	Con A-conditioned spleen cell supernatant
CSF	Colony stimulating factor
CsA	Cyclosporine
C.U.	Cytotoxic unit
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DTH	Delayed type hypersensitivity
EMEM	Eagle's Minimal Essential Medium
FD	FDc-P1 IL3 dependent cell line
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvHD	Graft versus host disease
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIFCS	Heat-inactivated foetal calf serum
IL1	Interleukin 1
IL2	Interleukin 2
IL3	Interleukin 3
γ-IFN	γ-Interferon
i.p.	Intraperitoneal

Ir	Immune response gene locus
LPS	Lipopolysaccharide
2-ME	2-mercaptoethanol
MAF	Macrophage activating factor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibition factor
MLR	Mixed lymphocyte reaction
O.D.	Optical density
PBS	Phosphate buffered saline
RPMI	RPMI 1640
S^+	Stimulating cell
S^-	non-stimulating cell
S.D.	Standard deviation
S.E.	Standard error of the mean
TCGF	T cell growth factor
UV	Ultraviolet
$\bar{X}$	Mean

CHAPTER 1

THE CELLULAR BASIS OF IMMUNE REACTIONS

1.1 THE CENTRAL PROBLEM

An important step in the scientific study of the immune system was taken by Pasteur in the late 19th century. As a consequence of his 'germ' theory of disease and the success of vaccination in preventing smallpox, it occurred to Pasteur that all 'germ'-related diseases could be prevented if attenuated 'germs' could be developed and used for immunisation (reported by Parish, 1965).

In the subsequent hundred years the art of vaccination has given way to the science of immunology. In fact, prevention of disease through vaccination need never impinge on the work of a modern day immunologist who can find convenient experimental systems in a number of diverse fields which are not necessarily related to disease control. A great deal is now known about the components and characteristics of the immune response. However, Pasteur's grand vision of protection against all infectious disease, has not been realised. One problem has been the difficulties associated with growing sufficient quantities of the organisms concerned, such as the causative agents of malaria and shistosomiasis. Future vaccines, therefore, depend on the development of alternative methods for preparing antigen suitable for immunization. Experience has already shown, however, that some antigen preparations can induce an immune response without providing significant protection against disease. One reason for this is that a response may proceed through a number of different pathways

which have diverse abilities in protection against certain diseases. This heterogeneity probably evolved to cope with infectious organisms which display different modes of pathogenesis. The basic features of this heterogeneity and some of the implications both for vaccination and as a scientific problem for analysis will be discussed below.

Adaptive immune responses (those that mount a faster and more effective response to secondary antigen challenge) fall naturally into two categories: immunity which can be transferred with serum (humoral) and immunity which is transferred with cells (cell-mediated immunity, CMI). Humoral immunity is a property of immunoglobulins, which in the mouse, exist in eight different forms or isotypes, seven of which are secreted and play a role in mediating humoral protection. Each isotype has a different capacity to interact with immune cell types and serum components. CMI, characterised by tissue graft rejection, virus clearance and delayed type hypersensitivity (DTH) reactions, is also heterogenous; the cells involved exist in at least two classes, each capable of at least two different effector functions.

To prepare an effective vaccine to a given infectious agent requires both a knowledge of the most effective immune response and the means by which that response can be induced. In many cases neither piece of information is available. Stimulating the appropriate immune response to prevent a given disease by immunization is the "central

problem" faced by vaccinators. A solution to this problem could be achieved entirely through experience with different antigen preparations, or, it could be solved through the development of a theory which would define the characteristics of antigen/immune system/immunization-protocol necessary to produce a nominated immune status.

The viewpoint adopted in this thesis is that future empirical vaccine development will be inferior to that based on a theoretical construction. Furthermore, one likely framework or paradigm within which a theoretical description could be formulated is the 'cellular' approach adopted by biologists to describe the working of a multicellular organism. Thus the work to be described here bears in no direct way on vaccination, but does explore one arm of the immune response in the theoretical terms defined by the cellular paradigm.

In the next section some general properties of cellular signalling will be described. Later sections will review some of the known features of the cells which contribute to the observed heterogeneity of the immune response.

1.2 CELLS, SIGNALS AND THE BIOLOGICAL RESPONSE

A philosophy which underlies most current work in immunology is the 'cellular paradigm'. It is by now unnecessary to point out that all animals are composed of individual units (cells) each carrying a full complement of

genetic material and each one "programmed" through a series of differentiative steps to respond in a particular way to its environment. An important principle of this paradigm is that many of the signals that alter the behaviour of each cell are received from the cell surface through a receptor.

Within any multicellular organism each cell can be defined in terms of this paradigm. The two basic components, receptor and programmed response, can be quite plastic: the same receptor does not necessarily trigger the same response in different cells, and quite different receptors on different cells may trigger a similar response. This simple yet powerful principle is a dominant force in attempts to explore and describe the workings of a multicellular organism. The success it will achieve is unknown. What is clear is that the methods of analysis it provokes are many years from being exhausted. Its application to the study of the immune system is in its infancy.

Before treating in detail the cells engaged in the immune system, it is necessary to discuss some general characteristics of cell signalling via membrane receptors. The relationship between the molecule that binds to the receptor (ie. pharmacon, ligand, drug), the receptor itself and the resulting biological response is given by the following scheme.

$$L + R \underset{K_2}{\overset{K_1}{\longleftrightarrow}} LR \xrightarrow{K_3} E$$

L is the ligand, R the receptor and E is the biological effect. The rate constants K_1 and K_2 determine the strength with which L and R bind. K_3, called the 'intrinsic activity' by Ariens (1954) and the 'efficacy' by Stephenson (1956) relates the number of L-R complexes present on the cell surface to the strength of the biological effect. It is possible to proceed and prepare quantitative expressions for each term. The exercise however is not necessarily general and will vary for different systems. However, a qualitative observation is relevant. That is, that receptor binding alone does not necessarily lead to cell triggering. This has been amply demonstrated with competitive inhibitors of drugs. These agents bind with high affinity and yet do not signal any biological effect. Thus, the second reaction, defined by K_3, must always be considered when describing the triggering requirements of a cell.

1.3 THE LYMPHOCYTE

The cell type responsible for both immunoglobulin production and CMI is the lymphocyte. To apply the cellular paradigm to the generation of response heterogeneity requires a restatement of the nature-nurture debate which occurs in the study of human behaviour:

> How much of the heterogeneity is a function of separate "ground-state" lymphocytes (that is lymphocytes which have not been exposed to antigen) and how much depends on changes to the cellular program resulting from signals received during or after antigen triggering?

One aspect of this question which can be adequately answered is how the lymphocyte can respond to so many different antigens - including molecules synthesized de novo in the laboratory. Is the information for such a diverse response coded in the germline (nature) or is it learned after antigen challenge (nuture) ? The answer in the case of immunoglobulin production has both a cellular and molecular component.

The cellular basis was proposed as the clonal selection theory by Burnet (1957). This theory holds that each lymphocyte expresses and makes antibody of only one specificity. This cell is "stimulated" following antigen exposure and proliferates and differentiates into a pool of cells each secreting antibody identical to that which was expressed by the original cell. This enlarged cell pool ensures a greater response to subsequent challenge with the same antigen. The explanation of how lymphocytes achieve sufficient diversity to respond to so many antigenic determinants has been provided by analysis of immunoglobulin genes.

The molecular genetics of antibody formation has been recently reviewed by Tonegawa (1983) and Honjo (1983). The immunoglobulin molecule is a peptide composed of two identical light chains and two identical heavy chains joined by disulphide bonds. The carboxy (C) terminal end of the light chain is relatively invariant within a species. The

C-terminal end of the heavy chain varies within a species along the lines of immunoglobulin class. That is, for example, all IgM molecules express the same C-terminal segment. The amino terminal portion of both light and heavy chains is highly variable. It is this variability that gives rise to the large number of available antibody specificities. Four mechanisms have been described which adequately explain the generation of diversity of the immunoglobulin variable (V) regions.

The first mechanism occurs via recombination. The immunoglobulin gene in lymphocytes contains a number of genetic building blocks many of which exist in a number of copies. For example, a light chain gene, Kappa, is put together by joining one of 300 possible germ-line encoded V_K genes with one of 4 germline J_K genes. The heavy chain is constructed from 1 of 200 germline V_H genes, 1 of 12 D genes and 1 of 4 J_H genes. The second mechanism of diversity occurs in the joining region between the building blocks. So called "junctional site" diversity occurs at the V_L-J_L, V_H-D and D-J_H junctions as the joining ends are imprecise. A third mechanism depends on the insertion of several nucleolides in the V_H-D and D-J_H junction. A high frequency of somatic mutation of bases throughout the variable region is the fourth mechanism for the generation of diversity. It is estimated by Honjo (1983) that 5×10^7 different variable regions could be created by the first three mechanisms alone.

1.4 LYMPHOCYTE GROUND STATES

Different ground state lymphocytes are assumed to be cells which are programmed to respond in different ways to their first antigenic exposure.

The first distinction which can be drawn is that between "T" and "B" lymphocytes. Miller (1961) and Martinez et al (1962) showed that removal of the thymus from neonates considerably weakened their ability as adults to reject skin grafts. Subsequent work demonstrated a role for thymus derived lymphocytes in other cell-mediated reactions such as DTH (Arnason et al, 1962) and recovery from virus infection (Blanden, 1971; Yap and Ada, 1978). It was shown by Davies et al, (1967) that thymus-dependent lymphocytes do not make antibody. A complementary experiment by Glick et al (1956) showed that removal of the avian Bursa of Fabricus from chickens within two weeks of birth severely compromised their ability to produce antibodies to S. typhimurium. It was later found that bursectomized chickens were fully competent to effect CMI reactions (Aspinal et al 1963).

Lymphocytes which mature in the thymus and mediate CMI reactions have been named "T" lymphocytes. Although mammals do not have a bursa, the lymphocytes which produce immunoglobulin are referred to as "B" lymphocytes. Their site of differentiation (which is presently unresolved) will be called the mammalian "bursa" equivalent.

TABLE 1.1

Biological activities of murine immunoglobulins[a]

Reaction	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
Complement fixation	+	-	+		-	-	-
Cutaneous anaphylaxis	-	-	+	+	-	-	+
Macrophage binding		-	+				
Placental transfer	-	+	+	+	+	-	-

[a] Adapted from Spiegelberg (1978) Adv. Immunol. 19: 259-294

Thus, CMI-inducing and immunoglobulin-secreting lymphocytes are physically distinct cell types with different "cell programs" laid down prior to antigen exposure as a result of differentiation in separate anatomical sites. The B cell antigen receptor is immunoglobulin. At least one component of the T cell receptor is an immunoglobulin-like heterodimer composed of an α and β chain. Both chains exhibit variable and constant regions. The basic properties described previously for the generation of immunoglobulin diversity, such as clonal selection and gene rearrangement (Section 1.3) also apply to the T cell receptor (reviewed by Hood et al, 1985).

1.5 LYMPHOCYTE FUNCTION

The effector function of B lymphocytes is mediated by secreted immunoglobulins. The seven secreted murine immunoglobulin isotypes important in immunity are listed in Table 1.1 along with their functional characteristics. As different immunoglobulin isotypes have different functional capacities the ability to generate a given isotype is clearly important. This isotype variation is not a ground-state property of B cells (reviewed by Cebra et al, 1984). Treatment of mice from birth with anti-μ (the IgM heavy chain antigenic determinant) prevented the appearance of not only IgM but all Ig isotypes (Manning and Jutila, 1972). This occurs despite the observation that B cells usually only express one surface isotype. This experiment suggested that B cells must first express IgM before making

IgG, IgE or IgA. The molecular biology of this B cell isotype switching has been described by Shimizu et al, 1982, but the antigenic and cellular requirements which lead to the switch are poorly understood. T lymphocyte involvement is important for the initation of secretion of particular isotypes from B cells, ie. IgG_1 (Taylor and Wortis, 1968; Torrigiani, 1972), IgE (Hamaoka et al, 1973; Ishizaka and Okudaira, 1973) and IgA (Torrigiani, 1972; Cebra et al, 1976).

The immunoglobulin isotype produced following antigen exposure is not predetermined but is influenced by a number of parameters. The first described is the effect of reimmunization, which for many antigens induces a switch from IgM production to IgG. The presentation of antigen associated with a particular adjuvant can also affect the developing isotype response. The clearest example of this phenomenon is the ability of alum precipitated antigen to induce an IgE response (Revoltella and Ovary, 1969). The physical characteristics of the antigen itself can play a role, for example potent B cell mitogens when injected into mice induce quite different patterns of immunoglobulin isotype production (McKearn et al, 1982). Another important variable is the route of administration. Rats primed to cholera toxoid by an intraperitoneal (i.p.) injection of antigen produce very few cells which release IgA if rechallenged i.p., however large numbers of IgA secreting

cells are detected if the second antigen dose is given intragastrically (Pierce and Gowans, 1975).

Possible mechanisms by which T lymphocytes initiate CMI reactions were discovered following the development of *in vitro* techniques for culturing lymphocytes. Bloom and Bennett (1966) described the release from sensitized lymphocytes of a molecule which inhibited the *in vitro* migration of macrophages. Other factors were described such as mitogenic factor, which triggered lymphocyte proliferation (Gordon and MacLean, 1965) and chemotactic factors which directed the *in vitro* movement of cells such as eosinophils (Cohen and Ward, 1971), macrophages (Ward, Renold and David, 1970) and basophils (Kay and Austen, 1972). The obvious parallels that these *in vitro* activities have with the cellular infiltration which characterises the DTH reaction, implied that this reaction occurred in response to the release of these factors. Injection of cell-free supernatants containing lymphocyte-derived factors produced a typical DTH lesion (Bennett and Bloom, 1968). The generic term "lymphokine" was coined by Dumonde *et al*, (1969) to include all immunologically active lymphocyte-derived factors other than immunoglobulin.

Another T cell function, cell mediated cytotoxicity, was first described by Brunner *et al*, (1970). Lymphocytes from the spleen, lymph nodes and blood from an animal inoculated with a foreign tumour, were found to specifically lyse homologous tumour cells *in vitro*. Cell-mediated

cytotoxicity is also active against virus-infected cells (Blanden et al, 1976) and cells carrying foreign minor histocompatibility antigens (section 1.6) (Bevan, 1976a).

The parameters which affect immunoglobulin isotype production described above for B cells, probably also affect T cell heterogeneity. Two examples are the immunization protocol for inducing DTH reactions and the infectious status of an immunising virus. Guinea pigs innoculated intradermally with vaccinia virus in saline develop on rechallenge, a DTH reaction where the predominant infiltrating cell type is the basophil. By contrast animals sensitized with the same dose of virus in complete Freunds adjuvant develop 'true' DTH, characterised histologically by the presence of numerous mononuclear cells and only rare basophils (Dvorak and Hirsch, 1971). The distinction in cell infiltrate probably reflects the elaboration of different lymphokines by the antigen-reactive lymphocytes. The second example is the greater effectiveness of live virus vaccines over killed virus preparations in providing protection against natural virus infection. A more detailed discussion of this phenomenon is included in section 1.8.

1.6 GRAFT REJECTION AND THE MAJOR HISTOCOMPATABILITY COMPLEX

Studies by Gorer et al, (1948) and Snell (1948) recognised that the fate of tumour grafts between non-identical mouse strains was controlled by at least 7 and possibly 14 genetic loci. These genes controlling

histocompatibility were designated "H" and numbered serially (Snell, 1948). Tissue rejection across the genetic disparity, "H-2" was particularly rapid. Thus the murine H-2 became known as the major histocompatibility complex (MHC). All other "H" loci were collectively referred to as minor or non-H-2 histocompatibility genes.

Today the protein products and organization of the MHC gene region are well described (Reviewed by Hood et al, 1983; Klein, et al, 1983). Three subregions of the MHC, K, D and I code for glycoproteins expressed at the cell surface. The K and D region products display a marked homology in both sequence and structure. Each molecule consists of a single polypeptide chain of molecular weight 45 000 daltons non-covalently associated on the cell surface with a small polypeptide called β-2-microglobulin (12 000 daltons). Cell surface molecules with these basic features were termed "class 1" MHC antigens by Klein (1979).

Two cell surface molecules have been identified as being coded for in the MHC I region. Both products are similar in structure and sequence and are known as class 2 MHC antigens (Klein, 1979). These molecules consist of 2 non-covalently linked chains called α and β. The α chains range in molecular weight from 30 000 to 33 000 daltons. Theβchain has a molecular weight of 28 000 daltons.

Class 1 and class 2 MHC products exist in many different allelic forms. The allelic composition of the MHC is termed the MHC haplotype and is designated by an alphabetical superscript, eg the DBA mouse strain is called H-2^d (made up of K^d, D^d and I^d). Recombinant mouse strains are available with haplotypes containing H-2 alleles from different strains.

Class 1 and class 2 MHC products display a different pattern of expression in tissues. In general, class 1 antigens can be found on all mammalian cells whereas the class 2 antigens are much more restricted, being present on B lymphocytes and other lymphopoietic cell types such as macrophages, dendritic cells (Steinman and Witner, 1978) and T cells (Hammerling, 1976). Class 2 antigens are also expressed at the cell surface of a number of cell types during an immune reaction (Mason et al, 1981).

One clue as to why the MHC products are particularly strong transplantation antigens was provided by the discovery of the mixed lymphocyte reaction (MLR). Incubating lymphoid cells from two strains with a disparity at the MHC, in in vitro culture results in a rapid proliferative response (Bain et al, 1964). It was estimated by Simonsen (1967) and Wilson, et al (1968) that 1-3% of all T lymphocytes are reactive to cells bearing foreign MHC antigens. No generally agreed explanation for this remarkable preoccupation of T lymphocytes with foreign MHC has yet been supplied, although a number of competing

theories based on the discovery of MHC restriction (next section) have been proposed. The high frequency of reactive cells does explain why these antigens are particularly important in triggering tissue graft rejection.

1.7 T CELL RESTRICTION

Although the H-2 loci were defined for their role in tissue graft rejection, subsequent studies have revealed a much broader influence of the MHC on T cell function. Two examples to be discussed below are MHC restriction of T cell killing and the control of the immune response to simple antigens.

T lymphocytes whch were cytotoxic for lymphocytic choriomeningitis virus - infected cells were found to kill only infected cells which shared the MHC region with the reacting lymphocytes (Zinkernagel and Doherty, 1974). This MHC "restriction" of effector function has been shown for many other viruses (reviewed by Zinkernagel and Doherty, 1979) as well as for cytotoxic cells which recognise minor histocompatibility antigens (Bevan, 1976b) and chemically altered target cells (Shearer, 1974). Other studies found that the amount of antibody produced by different mouse strains following priming with simple antigens such as poly-L-lysine and T(GAL) was controlled by the H-2 gene complex (McDevitt and Chinitz, 1969). Later mapping studies isolated this "immune response" (Ir) locus to the I region of the H-2 (McDevitt *et al*, 1972).

The currently accepted view of MHC-dependent immune phenomena is that they all occur as a result of the requirement for T cells to be restricted by one or other cell surface expressed MHC product (Blanden et al, 1975; Klein et al, 1981). This theory maintains that MHC gene products serve to guide T lymphocytes in distinguishing between self and non-self. The Ir - gene control of antibody levels is explained by the role of I region (or class 2) restricted T cells in helping B lymphocytes produce antibody. Evidence for this assertion is the observation that T cell proliferation in vitro to a given antigen correlates well with the level of antibody formed in vivo (Klein and Nagy, 1982).

It is not yet determined how the T cell antigen receptor detects MHC and antigen, nor is it clear how T cells become "restricted"; whether by a learning process in the thymus (ie. pre-antigen as suggested by Zinkernagel (1978))or as a result of the T cell's particular activation requirements (i.e. during antigen stimulation as suggested by Lafferty et al, 1983). It is clear however, that MHC restriction limits T cell function to cell-associated antigen.

Matzinger and Bevan (1977) have shown how MHC restriction provides an explanation for the high proportion of T cells reactive to foreign MHC-bearing cells. Their hypothesis holds that T cells recognize an 'interaction' antigen, formed by the close association of MHC and a cell

surface antigen. T cells reactive to self antigen-self MHC association are assumed to be in some way unresponsive. Incorporation of a foreign MHC molecule in to the cell membrane will allow interaction antigens to form with each 'self' cell surface component, which, by the rule of MHC restriction, will no longer resemble self and be potentially antigenic. As the number of non-MHC products on a cell surface is likely to be large, many new antigens will be generated. In contrast, incorporation of a single non-MHC antigen into the cell surface will create a smaller number of interaction antigens. This number will be related to the number of new antigens created by a foreign MHC product by a factor proportional to the number of different cell surface molecules.

1.8 T CELL ACTIVATION

Implicit in this discussion so far has been the assumption that T lymphocytes exist in at least two states: a "resting" form which will not contribute to an immune response, and an "active" form. It is the active form of the T cell which is cytotoxic and releases lymphokines. Clearly T cell "activation" is a necessary step before any CMI reaction can be generated.

Most information on T cell activation has come from studies <u>in vitro</u> using either alloantigenic cells to trigger an MLR or T cell mitogens (section 1.9) to stimulate large numbers of lymphocytes. Early studies showed that treating

lymphoid cells with ultraviolet (UV) radiation or heating the cells to 45^{o} for 60 minutes destroyed the cells' ability to initiate a MLR, without affecting the levels of serologically detectable MHC antigens (Lindahl-Keissling and Safwenburg 1972; Lafferty et al, 1974; Schendel and Bach 1975). These studies indicated that antigen alone was an insufficient stimulus to drive the proliferative and differentiative reaction which occur in an MLR. Further studies have shown that antigenic cell viability is not the sole requirement, as stimulating ability is restricted to only a few cell types, all of which are haemopoeitic in origin, and most of which carry class 2 MHC antigens (Sunshine et al, 1982; Minami et al, 1980). The correlation between stimulation ability and class 2 antigen expression is interesting although not absolute. A number of tumour cells will stimulate and are class 2 MHC negative (Lafferty et al, 1980) while B cell lymphomas express class 2 antigens and are unable to stimulate an MLR (Glimcher et al, 1982). Cells capable of triggering a primary MLR were designated S^{+} (for positive stimulation) by Lafferty and Cunningham (1975). S^{-} cells can induce a positive reaction if the cultures are supplemented with cell-free supernatants from mitogen stimulated T lymphocytes (Talmage et al, 1977). These supernatants without antigen will not stimulate T cell activation. This result suggests that S^{+} cells produce a factor which 'costimulates' the T cell response to antigen. One such costimulating factor, interleukin 1 (IL 1) is released by lipopolysaccharide (LPS) or activated T cell-

triggered macrophages and macrophage-derived tumour cell lines (Mizel et al, 1978; Farrar et al, 1980).

The concept that a special cell type is required to activate T cells suggested that these stimulator cells in tissue grafts represent the major barrier to successful transplantation. Lafferty et al, (1975), and Bowen et al, (1980) found that long-term culture of thyroid or pancreatic islet tissues in 95% O_2 and 5% CO_2 indefinitely prolonged survival of this tissue in allogenic hosts. If engrafted animals received an injection of donor-type spleen cells rapid rejection followed (Lafferty et al, 1980). The explanation proposed by Lafferty et al (1981) is that cultured tissue is free of S^+ cells and therefore stimulation of host graft-specific T cells cannot occur (antigen presented on host S^+ cells would trigger a host-MHC restricted response which would not be graft specific).

Further evidence implicating S^+ cells in this phenomenon has been provided by Faustman et al, (1981) who treated uncultured islets with anti-class 2 MHC antibody and complement and achieved a significant increase in graft survival.

The mode of antigen presentation plays a role in activation of different classes of T cell. A study by Ertl (1981) examined the properties of viruses which were important in generating class 1 or class 2 - MHC restricted T cell responses. T cells primed in vivo with infectious

sendai virus were restimulated for 5 days *in vitro* with different preparations of virus and different antigen presenting cell types. The resulting cells were tested for their ability to induce DTH. Class 1 - MHC restricted DTH occurred only if the virus preparation was infectious or could fuse with the cell membrane. Class 2 restricted DTH - inducing T cells were only stimulated by macrophages from the spleen or peritoneal cavity. In this case virus infectivity was not required. The general conclusion to be drawn from this work is that class 1 restricted T cells are only triggered by antigen which can incorporate as part of the cell membrane. Conversely, antigen which cannot be incorporated into the cell membrane must be 'processed' by a specialized cell type (see section 1.11). This mechanism leads to class 2 - MHC restricted T cell responses. The reason for this division, in cellular or molecular terms, is unknown.

Studies by Leung and Ada (1981) have shown that class 1 restricted T cells are much more effective in clearing influenza virus from infected mouse lung than are class 2 restricted T cells. In fact these latter cells contribute to the observed lung pathology. Clearly in considering anti-viral vaccines it would be advantageous to understand the mechanism by which a class 1 - MHC restricted response can be generated to the exclusion of a class 2 restricted response.

1.9 T CELL ACTIVATION: MITOGENS

Nowell (1960) first showed that plant lectins (multivalent sugar-binding glycoproteins), could initiate lymphocyte proliferation. This "mitogenic" response could be either T lymphocyte or B lymphocyte specific depending on the lectin used (Jannossy and Greaves, 1971). This ability of plant lectins to distinguish between T and B lymphocytes suggests that the triggering mechanism used by the two cell types are different. A number of mitogenic lectins have now been described. The differences between mitogenic lectins and non-mitogenic lectins is assumed to reflect the binding of different cell surface glycoproteins (Dillner-Centerlind et al, 1980).

In 1971 Novogrodsky and Katchalski reported that mild oxidation of lymphocytes with $NaIO_4$ induces extensive proliferation of T lymphocytes. A major cell surface change caused by this agent is the conversion of sialic acid to an aldehyde (Van Lentan and Ashwell, 1971). These aldehydes were implicated in the triggering process following the demonstration that the aldehyde blocking agents KBH_4 and NH_2OH markedly reduced the response (Novogrodsky and Katchalski, 1972; Zatz et al, 1972). Sequential treatment of lymphocytes with neuraminadase and galactose oxidase, which also generates aldehyde moieties is also mitogenic (Novogrodsky and Katachalski, 1973).

T cell activation by lectins was found to require the participation of class 2 MHC bearing accessory cells (Habu and Raff, 1977 ; Ahmann et al, 1978). Thus "mitogenic" lectins are not directly mitogenic for lymphocytes.

Larsson and Coutinho (1980) showed that accessory cell requirement could be replaced by cell-free supernatants from lectin stimulated spleen cells. Similarly $NaIO_4$ stimulation was found to require "accessory cells" which can be replaced with soluble factors (Greineder and Rosenthal, 1975).

These experiments showed that mitogen stimulation displayed requirements identical to those found for alloantigen stimulation. Apparently a number of steps are involved in T lymphocyte activation. Curiously, stimuli as diverse as lectins, cell oxidation and cells carrying foreign MHC fulfill these requirements for a large percentage of T lymphocytes.

1.10 PROPERTIES OF ACTIVATED T CELLS

Once activated by antigen or mitogen, T cells express receptors for a lymphokine which triggers growth of these cells. This lymphokine, T cell growth factor (TCGF), has been used to maintain activated T cells in culture indefinitely and to clone T cells of a particular specificity (Shreier et al, 1980; Nabholz et al, 1978; Gillis and Smith, 1977). TCGF was given the "neutral" term interleukin 2 (Aarden et al, 1979) (IL2) when it was found to possess other biological activities such as the ability

to influence B cell differentiation (Watson et al, 1979) as well as costimulating the response of thymocytes to mitogens (Mills et al, 1976). Another method found for immortalising a T cell is to fuse activated T cells with a tumour cell. The resulting hybridomas grow independently of IL2, yet display a number of T cell functions, such as lymphokine release (Kappler et al, 1981). The requirements for triggering lymphokine release from IL2-dependent T cells or T cell hybridomas are much simpler than those found for T cell activation. Alloreactive T cells are triggered by any cell bearing the appropriate antigen, including cells rendered metabolically inactive by UV irradiation (Andrus and Lafferty, 1981). Also, cloned T cells can be triggered to lymphokine production by T cell mitogens, in the absence of the accessory cells found to be obligatory for activation by these agents (Ely et al, 1981).

The number of different lymphokines which can be released by T cells is unknown. Waksman (1979) documented 100 different activities characterized by an assay and an acronym. This number is probably an order of magnitude too high as many different assays have been found to measure the same activity. For example, macrophage activation factor (MAF), which increases the ability of macrophages to kill tumour cells, and γ-interferon (γ-IFN), which inhibits virus replication, are probably the same lymphokine (Pace et al, 1983). Also, interleukin-3 (IL3), originally defined by its ability to induce an enzyme 20α hydroxysteroid

dehydrogenase in spleen cells of nude mice (Ihle, et al, 1981) was found to be an active growth factor for mast cells as well as causing growth of bone-marrow cells in agar (Ihle et al, 1983).

Potentially, lymphokine producing and cytotoxic T cells could reflect separate ground states or mutually exclusive cell programs. However, both possibilities are ruled out by the demonstration that T cell clones can exhibit both functions (Kelso and Glasebrook, 1984; Morris et al, 1982; Dennert et al, 1981).

Cytotoxicity is generally associated with class 1 MHC restricted cells, although sufficient numbers of cytotoxic class 2 restricted cells have been described to prevent a generalization (Wagner et al 1975; Klein et al, 1977; Spits et al, 1983; Kaplan et al, 1984). One non-functional phenotypic characteristic, which does appear to be "absolute" is that class 1 MHC restricted T cells express the lyt 2 marker - defined initially by Cantor and Boyse (1975) as a marker for the cytotoxic function. Recently a murine marker for class 2 restricted T cells, called L3T4, has been described (Dialynas et al, 1983).

A number of authors have examined T cell clones for their lymphokine release and cytotoxic potential. Kelso et al, (1982) found from a study of 72 individual clones, reactive to either Moloney leukaemia virus, minor histocompatability antigens or MHC antigens, that MAF was

released by 68 clones and that release of this activity did not correlate with their cytolytic activity or with production of other lymphokines such as IL2 or a colony stimulating factor (CSF) for granulocyte-macrophages (GM-CSF). Most IL2 releasing cells were found to be non-cytolytic but there were exceptions. All lymphokines achieved a maximum activity within 24 hours of antigen stimulation. In a later report Kelso and MacDonald (1982) looked at the precursor frequency of cytolytic and lymphokine releasing cells. As a starting point these authors used a MLR with limiting numbers of responder cells selected on the basis of lyt 2^+ or lyt2^- phenotype and incubated for 7-9 days. The resulting clones were split and restimulated for lymphokine production and tested for cytotoxicity. All cytotoxic cells were found to be derived from lyt2^+ precursors. This result may be meaningless as the target cell used, P815, carries only class 1 MHC antigen, and therefore would not be recognized by the class 2 reactive (Lyt2^- population). Precursors of cells secreting MAF and GM-CSF were present in both lyt2^+ and 2^- populations. IL2 release was found predominantly in the lyt2^- non-cytotoxic population. A similar study using the mitogenic lectin conconavalin A (Con A) to provide the primary stimulus reached similar conclusions; ie. T cells are heterogenous in their ability to secrete a variety of lymphokines irrespective of their lyt phenotype (Guerne et al, 1984). In this work 50% of lyt 2^+ clones produced IL2 - a much higher percentage than that reported by Kelso and

MacDonald, (1982). A further characteristic of lymphokine release revealed from analysis of clones is that the range and amount of lymphokine produced per clone is constant (Kelso and Glasebrook, 1984). An exception to this rule is IL2 release which was seen to decrease with time in an unpredictable manner.

These studies highlight the potential heterogeneity of T cell effector function. There appears to be little correlation between the "repertoire" of lymphokines a T cell clone will produce, the cytotoxic capacity of the cell and the cell surface phenotype and MHC restriction class.

1.11 ANTIGEN PRESENTING CELLS, STIMULATING CELLS, ACCESSORY CELLS.

The need for "costimulation" of a primary T cell response and the MHC restriction of T cells gives rise to a requirement for accessory cells in culture. These cells could be defined as non-responding cells which must be included with T cells before a response can be generated. From the discussion in the previous sections of this chapter some features of this accessory cell requirement can be deduced.

For a MHC-restricted T cell to respond to a soluble protein then that protein must be "presented" on the surface of a cell bearing the appropriate MHC antigen. Studies by Ziegler and Unanue (1981) have shown that this presentation

is the function of macrophages and requires an active internal processing step which is inhibited by chloroquine.

Glimcher et al, (1982) have reported that B cell tumours can process soluble antigen for recognition by T cells but cannot stimulate a primary response to alloantigen. Thus "processing" cells can be divided into two classes, those that are S^+ and could therefore stimulate a primary response to soluble or processed antigens, and those that are S^-. S^- antigen presenting cells would be capable of triggering lymphokine release from an activated T cell.

If the antigen is not soluble and is present as a cell-surface component (as follows infection with virus) then any cell expressing the antigen plus MHC could presumably present the antigen. That is, in this case there is no requirement for a specialized antigen processing function. Presumably this class of antigen must be present on the surface of an S^+ cell to activate reactive T cells. These categories of accessory cell, stimulating and non-stimulating, processing and non-processing, are often confused in the literature and require a more formal universal nomenclature.

1.12 CONCLUSIONS

The intention of this introduction was to review briefly the cellular foundation for the heterogenous nature of the immune response. This heterogeneity is a function of different lymphocyte classes which have the potential for alternative effector programs. B lymphocytes may secrete any one of seven immunoglobulins, whereas T lymphocytes secrete a range of lymphokines selected from a large set of possible activities. T lymphocytes may also kill antigen bearing target cells. The features which control this diversity in cell function are poorly understood, however, parameters such as route of administration, dose, and frequency of antigen exposure all appear to play some role. The ability of these external factors to influence the class of an immune reaction imply that a reaction is not predetermined or preprogrammed to respond in a particular way to a given antigen determinant. Rather these observations suggest a general view that the ground state lymphocyte program and those programs which unfold during the immune response are patterned to fit not the antigen, but the nature of the antigen, its mode of presentation and the site of exposure. The conclusion is inescapable that, under normal circumstances, a response appears to fit the antigen challenge. For example, the lymphocyte generated during live virus (but not killed virus) infection, is cytotoxic, a response which appears "appropriate" to cope with a virus infection. It seems likely that similar 'appropriate' responses have evolved to counter other

antigen classes. To avoid arguing from final causes this concept can be rephrased into the more practical formulation of the vaccinator: what are the properties of each 'antigen class' which are perceived by lymphocytes and which ultimately influence the generation of different immune reactions?

The cellular paradigm can identify three broad mechanisms which would allow the lymphocyte to detect different antigen classes:

1 Lymphocytes could exist which have different antigenic triggering requirements. This parameter is difficult to quantitate, but relates to the efficacy of ligand-receptor interaction described in section 1.2. The number of possible mechanisms and the vagueness of the concept when applied to lymphocytes (for instance the term "cross-linking" is used by immunologists to describe a triggering requirement) suggests that the pharmacological term "efficacy" in general use for drugs is inadequate for the use of immunologists. An early now abandoned term "intrinsic activity" coined by Ariens (1954) could be used in this context.

2 Lymphocytes could be influenced by extra 'hormonal' signals received during antigen stimulation. This mechanism has been shown to be important for the activation of T cells. It could also carry information regarding the site of antigen exposure. For instance

the S^+ cell found in the skin, the langerhans cell, could "costimulate" a T cell in a different manner than other S^+ cells. This information could potentially then be expressed as a separate lymphokine repertoire in the activated T cell.

3 Lymphocytes could receive differentiative signals through cell surface molecules following contact with other cells. A documented case is the "cognate" signal received by B cells via their cell surface class 2 antigens following interaction with a class 2-restricted T cell (Howard and Paul, 1983). Clearly, this mechanism could transfer extra information to the B cell (ie. that a class 2-restricted T cell response can be mounted against the antigen which the B cell recognizes).

These parameters represent the "solution" to the central problem outlined in section 1.1, in the sense that they could be shuffled into an algorithm for each class of immune response. It is important to recognize that this solution relies upon a philosophy, the cellular paradigm, which may or may not be appropriate for this problem. However, as mentioned previously, the success this method will achieve is unknown, while the experimentation it suggests is many years from being exhausted.

1.13 AIMS AND OUTLINE OF THIS THESIS

One problem which arises when studying the T lymphocyte antigen signal is the complex behaviour of conventional *in vitro* assay systems such as the MLR and mitogen stimulation. These systems do depend on lymphocyte signalling, however, other reactions involving cell interaction and release of soluble factors are also required. In this thesis we have attempted to 'isolate' the T cell antigen signal by measuring the induction of lymphokine release from activated T cell.

Chapter 3 establishes conditions whereby lymphokine release can be assumed to be dependent solely on antigen signalling. This chapter provides evidence that release of IL2 and IL3 are under identical control and therefore either lymphokine could be used to detect antigen signalling. IL3 is more generally useful as the assay is more sensitive and not subject to an inhibitor which can completely mask the presence of IL2.

Chapter 4 explores the effect of an immunosuppressive drug, cyclosporine (CsA), on the activity of activated T cells. This study isolates the drug's inhibitory action to a site after antigen binding and before mobilization of lymphokine-incoding genes - a site operationally defined as the T cell antigen signal. The specific site of action suggests CsA can be used to examine the role of the T cell antigen signal and lymphokine release both *in vitro* and *in vivo*. This potential is exploited in Chapter 5, where the

release function of activated T cells is found to be important for a number of lyt 2^+ cell dependent immune reactions.

Chapters 6 and 7 use the technique of dose response curve analysis to define the cellular requirements for signalling by antigenic cells and by T cell mitogens. These cellular requirements are complex and suggest; 1) that mitogens do not trigger the antigen signal by a direct action on the T cell; and 2) that the T cell antigen signal is an all or none event.

In the final chapter these findings are discussed for their relevance to the development of assays for activated T cells, and to the implications for the development of T cell response heterogeneity.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

The inbred mouse strains listed in Table 2.1, and a Canberra outbred strain were maintained in the Animal Breeding Establishment of the John Curtin School of Medical Research. Donors of lymph node and spleen cells were used between 6 and 14 weeks of age.

2.2 TISSUE CULTURE MEDIA AND BUFFER SOLUTIONS

Eagle's Minimal Essential Medium (EMEM) was prepared by dissolving 10 g of medium powder (GIBCO, 410-1500) in 1 litre of double distilled deionized water containing 2.2 g of sodium bicarbonate.

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by dissolving 10 g of medium powder (GIBCO, 430-1600) in 1 litre of double distilled deionized water containing 3.7 g of sodium bicarbonate.

RPMI 1640 (RPMI) was prepared by dissolving 10.4 g of medium powder (GIBCO) in 1 litre of double distilled deionized water containing 0.02% w/v sodium bicarbonate.

Hanks Balanced Salt Solution (HBSS) was prepared according to the procedure described by Hanks and Wallace (1949) and buffered with sodium bicarbonate at 0.013 g per 100 ml.

EMEM, DMEM and RPMI were supplemented with antibiotics (penicillin, 100 u/ml. streptomycin, 100 µg/ml, neomycin, 100 µg/ml) and sterilised by filtration through a 0.22 µm membrane (Millipore Corporation). HBSS was sterilized by autoclaving and antibiotics were added immediately before use. 2-mercaptoethanol (2-ME; KODAK) was stored at -20°C as a 0.1M solution in water. This stock was diluted 1/1000 to yield a final concentration in media of 10^{-4}M.

Heat inactivated foetal calf serum (HIFCS) was prepared by incubating selected serum batches (Flow Laboratories, Stanmore, Australia, and Commonwealth Serum Laboratories, Melbourne, Australia) at 56°C for 30 mins.

Phosphate Buffered Saline (PBS) was prepared according to the following formula: sodium chloride, 8.0 g; potassium chloride, 0.4 g; disodium hydrogen phosphate, 1.2 g; sodium dihydrogen phosphate, 0.39 g; double distilled deionized water, 1 litre.

Citrate buffer, pH 5.0 was prepared by mixing 205 ml 0.1M citric acid with 295 ml 0.1M sodium citrate and adding 500 ml distilled water.

Glycine buffer, pH 10.4 was prepared by mixing 250 ml 0.2M glycine with 193 ml 0.2M NaOH and adding 557 ml distilled water.

2.3 DETERMINATION OF CELL VIABILITY BY TRYPAN BLUE EXCLUSION

To determine viable cell numbers in preparations of normal or transformed cells, 0.1 ml of cell suspension was mixed with a known volume of a 0.5% w/v solution of Trypan Blue in PBS. An aliquot was then counted using a haemocytometer.

2.4 TRANSFORMED CELL LINES

Six tumour cell lines were used in this study. The strain of origin and characteristics of each, as well as the standard culture medium used in their maintenance, are given in Table 2.2. 5×10^4 viable cells were subcultured every 3-4 days into 25 cm^2 tissue culture flasks (Falcon, 3013 or Corning, 25100) containing 5 ml of culture medium. The flasks were then incubated at 37°C in a humidified atmosphere of 10% CO_2 in air.

2.5 PREPARATION OF LYMPHOID CELL POPULATIONS

Mouse spleen and lymph node cell suspensions were prepared by removing the organs aseptically, trimming away excess fat, and pressing them through a fine stainless steel grid into HBSS. The resultant cell suspension was transferred to a sterile centrifuge tube and allowed to settle on ice for 2 mins to remove clumps. The supernatant

was transferred to a fresh sterile centrifuge tube, centrifuged at 300g for 5 mins and the cell pellet was resuspended in EMEM containing 10% v/v HIFCS and 10^{-4}M 2-ME. The viability of the resultant single cell suspensions as assessed by Trypan Blue exclusion, ranged between 75 and 90%.

2.6 PREPARATION OF CONCANAVALIN A-ACTIVATED SPLEEN CELL SUPERNATANTS (CS)

CS was prepared using a modification of the method described by Talmage et al (1977). Spleen cells were cultured in serum-free EMEM containing 10^{-4}M 2-ME and 5 µg/ml concanavalin A (Con A)(Sigma, grade IV). Cultures were set up in 75 cm^2 tissue culture flasks (Falcon, 3024) with each flask containing 15 mls of medium and 3×10^8 spleen cells. These cultures were gassed with a 7% O_2, 10% CO_2 and 83% N_2 gas mixture, and incubated at 37°C for 2 hours. By this time, the cells formed a monolayer on the plastic surface. The original medium was discarded, and the adherent cells were washed gently 3 times with 15 ml of HBSS to remove unbound Con A. The cultures were then refed with 30 ml of serum-free EMEM containing 10^{-4}M 2-ME. The flasks were regassed and incubated at 37°C for a further 16-20 hours. The resulting supernatant was centrifuged for 10 minutes at 1800g to remove cell debris. The cell free supernatant was then concentrated 10-fold over a PM-10 membrane (Amicon), sterilized by filtration through a 0.22 µm Millipore membrane and stored at -20°C.

2.7 IRRADIATION OF CELLS

Normal splenocytes used as stimulators in mixed lymphocyte cultures were γ-irradiated with 750 rads from a ^{60}Co source. UV-irradiation of tumour cells or lymphocytes was carried out in 4 inch plastic petri dishes (Kayline, Adelaide) with the cell suspension ($\leq 10^6$ cells/ml) covering a depth of 1mm. Exposure was for 4 mins from a Sylvania G30TS Germicidal lamp at an intensity of 960μW/cm^2 in the 230-270 nm range.

2.8 MIXED LYMPHOCYTE CULTURES

The conditions for obtaining optimal responses in these cultures were taken from Talmage et al (1977).

The responding cell population was prepared from a number of pooled mesenteric lymph nodes. Suspended in EMEM containing 10% HIFCS and 10^{-4}M 2ME, 2 x 10^6 responding cells were added to each well of a 24 well tissue culture tray (LINBRO, 76-033-05). To these cells was added another 1 ml of medium containing 3.16 x 10^6 γ-irradiated spleen cells of the appropriate stimulating strain. Culture trays were incubated at 37°C for 4 days in a humidified atmosphere of 7% O_2: 10% CO_2: 83% N_2.

2.9 PREPARATION OF EXPANDED POPULATIONS OF ACTIVATED T-CELLS (SEVEN DAY ACTIVATED T CELLS)

Cells harvested from 4-day old mixed lymphocyte cultures

were diluted to 5 x 10^4/ml in EMEM with 10% HIFCS, 10^{-4}M 2-ME and 3-10% v/v CS. Cultures were incubated for 3 days at 37°C in 75 cm^2 tissue culture flasks (Falcon, 3024) in a humidified atmosphere of 7% O_2: 10% CO_2 : 83% N_2.

Cells prepared in this manner are >99% Thy 1 positive and contain less than 1.5% immunoglobulin positive cells (Woolnough and Lafferty, 1979).

2.10 IL2 ASSAY

The ability of IL2 to maintain the proliferation of Con A activated lymphocytes forms the basis of this assay.

Con A activated lymphocytes were prepared by incubating 10^6 C57Bl/6J (B6) spleen cells with 1 µg/ml Con A in 1 ml EMEM containing 10% v/v HIFCS and 10^{-4}M 2-ME. Cultures were set up in 24 well tissue culture plates (Falcon, 3008). On the third day of culture, the activated cells were harvested, washed once, and resuspended at 4 x 10^5 viable cells/ml in EMEM containing 10% v/v HIFCS and 10^{-4}M 2-ME.

Assays were prepared in flat bottomed 96 well trays (NUNC; 1-67008). Serial two-fold dilutions of the lymphokine preparation were made in 50 µl of EMEM containing 10% v/v HIFCS and 10^{-4}M 2-ME. All titrations were carried out in duplicate. Each well received 50 µl of the Con A activated

lymphocyte suspension. Control cultures consisted of 24 replicate wells containing 50 µl of cell suspension and 50 µl of medium. The cultures were then incubated at 37°C in a humidified atmosphere of 10% CO_2: 7% O_2: 83% N_2. After 16 hours, 1.25 µCi of ^{3}H-methyl thymidine (Amersham Intl. Ltd, TRK.418) were added to each well in 50 µl of HBSS, and the cultures were incubated for a further 5 hours. The cultures were harvested onto glass fibre filter paper discs (Whatman, Grade GF/A) using a Titertek Cell Harvester. The amount of thymidine incorporated by the cells in the culture was determined by placing the discs into a xylene based scintillation fluid and counting on a Packard liquid scintillation spectrophotometer. Figure 2.1 shows a typical dose response curve obtained from plotting $\log_{10}$ counts per minute (c.p.m.) per culture against the $\log_{10}$ lymphokine reciprocal dilution. Although sigmoid in shape, the curve approximates a straight line over its central region. The endpoint titre is obtained from this plot by measuring the dilution at which the straight line portion of the curve intersects the background level, set arbitrarily at 3 standard deviations (S.D.) above the mean ($\bar{X}$) c.p.m. of 24 control cultures. This assay is highly reproducible; the mean titre of duplicate titrations has a 95% confidence interval of $\pm$ 0.13 $\log_{10}$ units (Lafferty et al, 1980). Where IL2 titres are to be compared between different lymphokine preparations, all assays are performed on the same day.

2.11 MAINTENANCE OF IL3-DEPENDENT CELL LINES

IL3 is a haemopoietic growth factor which can be used to establish cell lines whose continued proliferation depends upon the presence of the lymphokine. These cells form the basis of a sensitive method for detecting and quantitating IL3 activity.

Two cell lines, 32D-cl (32D)(Greenberrer et al, 1983) and FDC-P1 (FD)(Dexter et al, 1980) were used in these studies.

A convenient source of IL3 for maintenance of these cells is the 24 hour supernatant obtained from the WEHI 3 tumour cell line. Both FD and 32D cells were routinely passaged in RPMI containing 5% HIFCS and 10% WEHI 3 supernatant.

2.12 IL3 ASSAY

Twelve serial two-fold dilutions of the test supernatant were prepared in 96 well microtitre trays (NUNC) using RPMI plus 5% HIFCS as diluent and yielding a final volume per well of 50 µl. Each well then received 2×10^4 FD (or 32D) cells in 50 µl RPMI plus 5% HIFCS. Trays were incubated at 37^oC in a humidified atmosphere containing 10% CO_2 in air. One of two methods was then used to measure the extent of proliferation of the IL3 sensitive cell line.

A) Tritiated thymidine incorporation: An aliquot of 1.25 µCi ^{3}H-methyl thymidine was added to each well after 16 hours of

culture and following a further 6 hour incubation, the thymidine uptake by the cells in each well was determined using the procedure described in section 2.10 for IL2 assays.

A typical plot of $\log_{10}$ c.p.m. versus $\log_{10}$ reciprocal supernatant dilution is shown in Figure 2.2. Endpoint titres were determined by fitting a line to the linear portion of the curve and reading the dilution where this line intersects with the background, defined arbitrarily as the mean plus 3 x S.D. of 24 control wells.

An estimate of the variability of this assay using analysis of variance was performed by assaying a standard preparation of IL3 activity in 6 replicates on 3 separate days.

The result of this study is presented in Table 2.3. Between day variation is sufficiently great to prevent comparisons to be made on assays performed on separate days. Removing the day to day variation illustrates the highly reproducible nature of this assay. The expected standard error (S.E.) on a duplicate assay is 0.074 providing a 95% confidence interval of ± 0.148 $\log_{10}$ units.

B) Measurement of hexosaminidase levels: To simplify the estimation of IL3 activity when large numbers of assays were required, the method of Landegren (1984) was employed. In

this procedure levels of the ubiquitous cell lysosomal enzyme, hexosaminidase, are determined to indicate the extent of cell proliferation per well.

The substrate p-nitrophenol-N-acetyl-β-D-glucosaminide (Sigma, N-9376) was dissolved at 7.5 mM in 0.1M citrate buffer, pH 5.0. The solution was then mixed with an equal volume of 0.5% Triton X-100 in water, and stored at -20°C

Each culture tray containing an IL3 assay was incubated for 48 hours and then centrifuged at 800g for 5 mins. The supernatant was removed by rapidly inverting the plate. Each well was then washed by adding 200 µl PBS, centifuging at 800g and again flicking off the supernatant. Washing was essential to remove traces of HIFCS which contain high levels of hexosaminidase. Each well then received 60 µl of substrate solution and the reaction was allowed to proceed for 1 hour at 37°C in a humidified atmosphere. The reaction was blocked and the colour developed by the addition to each well of 90 µl of a 50 µM glycine buffer, pH 10.4, containing 5 mM EDTA.

The absorbance of each well at 410 nm was measured in a Dynatech MR 600 microplate reader. Plots of $\log_{10}$ of optical density (O.D.) versus $\log_{10}$ supernatant dilution produced curves similar to that depicted in Figure 2.3. The intersection between a line drawn through the linear portion of the curve and the background O.D., defined arbitrarily as

the mean plus 3 x S.D. of 24 control wells containing medium and cells only, was taken as the endpoint titre.

The reproducibility of this assay was measured in a manner similar to that for the ^{3}H-methyl thymidine incorporation assay (section 2.12B).

The within day variation, shown in Table 2.4 is greater than that obtained for the thymidine uptake method. Thus duplicate determinations provide a 95% confidence interval of 0.193 $\log_{10}$ units. However the ease of this assay means that a greater number of replicates can be performed. Four replicates for example have a 95% confidence interval of 0.14, equivalent to duplicate samples in the thymidine assay.

2.13 ASSAY OF CYTOTOXIC ACTIVITY

R1(TL+) or P815 tumour cells at 5 x 10^6/ml in EMEM plus 5% HIFCS were incubated with 100 μCi/ml $Na^{51}CrO_4$ (Amersham Intl. Ltd) for 1 hour at 37°C. The labelled cells were then washed with 3 changes of EMEM plus 5% HIFCS and resuspensed to a final concentration of 10^6 viable cells per ml in EMEM plus 10% HIFCS.

Cell populations to be tested for cytotoxic activity were prepared in EMEM containing 10% HIFCS and a number of 2-fold dilutions in quadruplicate were prepared in flat

bottom microtitre trays (NUNC, 1-60078) in a final volume of 0.1 ml EMEM plus 10% HIFCS. Each well then received 0.1 ml labelled target cell suspension. Spontaneous ^{51}Cr release was determined by mixing 0.1 ml medium and 0.1 ml target cell suspension in quadruplicate cultures. Total releasable chromium was determined by mixing 0.1 ml target cell suspension with 0.9 ml distilled water in quadruplicate 5 ml plastic centrifuge tubes. All cell mixtures were incubated for 4 hours at $37^{o}C$ in a humidified atmosphere of 10% CO_2 in air. At the end of this period 0.1 ml supernatant was decanted from each well without disturbing the cells. Water lysis tubes were vortexed, centrifuged at 1200g for 5 mins and 0.5 ml supernatant removed. Supernatants were counted for 1 minute in a gamma scintillation spectrophotometer.

Cytotoxic activity is expresed in terms of cytotoxic units (C.U.), where one C.U. is the activity required to lyse one target cell under the conditions of the assay:

$$C.U. = -Y_o.\ln(1-y)$$

where Y_o = number of targets per asay well

y = the fraction of targets specificlly lysed during the assay

$$= \frac{\text{(test lysis - spontaneous lysis)}}{\text{(water lysis - spontaneous lysis)}}$$

The derivation of the cytotoxic unit has been described in detail (Woolnough and Lafferty, 1979).

2.14 STATISTICAL METHODS

Analysis of variance is used to gain an estimate of the variation of lymphokine release assays within and between days. This information is also used to ascribe confidence intervals on small mumbers of replicates (1 or 2). To do this, the "within day" variance is taken as an estimate of the assay variance. Thus the S.E. of the mean of 2 samples is given by:

$$\text{S.E.} = \frac{(\text{within day variance})^{\frac{1}{2}}}{\sqrt{2}} .$$

The t-distribution is then used to determine 95% confidence intervals, although, at the number of degrees of freedom used, this distribution is essentially normal and the 95% confidence limits are given approximately by the sample mean ± 2 x S.E.

It should be noted that this procedure assumes that the distribution of $\log_{10}$ endpoint titres is normal about the mean, that the standard deviation is independent of the size of the titre and that the variation observed on the original days of assay is preserved in subsequent assays.

2.15 ISOLATION OF ADULT MOUSE PANCREATIC ISLETS

Groups of 8-12 donor BALB/c mice were anaethetized with ether and sacrificed by cervical dislocation. The pancreas was removed, finely diced with scissors and washed in isolation medium - HBSS buffered with 0.5 g N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES)(Sigma)/100 ml and supplemented with Bovine Serum Albumin (0.15%), DNase (Sigma Type 1, 0.01 mg/ml) and antibiotics (penicillin, 100 units/ml, streptomycin, 100 µg/ml and neomycin, 100 µg/ml). The minced tissue was allowed to settle at 4°C for 4 mins and debris remaining in suspension was removed with a Pasteur pipette. After three 30 second-dicings and subsequent washings, the minced tissue was transferred to a siliconized 15 ml graduated glass centrifuge tube and allowed to settle for an additional 4 mins. The volume of tissue was noted and the tissue was then transferred to a siliconized 20 ml glass vial. Collagenase (Boehringer Mannheim) was freshly prepared in PBS at a concentration of 10 mg/ml, and then filtered through a 0.20 µm filter (Millipore Corporation). One ml of this solution was added to each 1 ml volume of minced pancreatic tissue and the volume was made up to 5 ml with isolation medium. The pancreatic tissue was then digested at 37°C for 17 mins in a shaking water bath. The digest was vigorously shaken by hand for an additional 15-20 seconds, in order to disperse any residual clumps of tissue. The enzymatic digestion was arrested by the addition of 15 ml of chilled isolation

medium and the digest was then allowed to settle for 4 minutes at 4°C. The supernatant was removed, and the digested tissue was washed twice with isolation medium. Individual islets were identified using a stereomicroscope set at 15 x magnification and then removed from the digest with a fine siliconized pipette. The isolated islets were placed on ice in a petri dish containing RPMI culture medium supplemented with HIFCS. The isolated islets were kept on ice prior to preparation for organ culture.

2.16 ORGAN CULTURE OF ADULT MOUSE PANCREATIC ISLETS

Adult islets were cultured as islet clusters. Groups of approximately 50 islets were transferred to 35 mm hydrophobic petri dishes (Falcon, 1008) containing 2 ml of RPMI culture medium supplemented with 10% HIFCS. To promote islet aggregation, initially each group of islets was gently rotated on an orbital platform (gallenkamp SGM-300, ENGLAND) at 50-60 cycles per minute, for 18-24 hours. The cultures were maintained at 37°C in a humidified gas phase of 95% O_2, 5% CO_2. The islet aggregates were then maintained as stationary cultures for 7 days in the same gas phase. The culture medium was changed 3 times per week; on the first and subsequent changes, the culture medium was removed using a Pasteur pipette and 0.75 ml fresh culture medium was replaced in each dish. During the culture period each aggregate condensed into a tight cluster suitable for transplantation.

2.17 PREPARATION OF AVERTIN ANAESTHETIC

Avertin was prepared by dissolving 1.0 g of 2,2,2 tribromoethanol (Fluka AG, Switzerland). The solution was diluted to a final volume of 50 ml with hot tap water (50°C) and shaken vigorously to mix. The v/w dose of avertin used to anaethetize mice was 0.010 - 0.013 ml/gm body weight, administered intraperitoneally (i.p.).

2.18 TRANSPLANTATION UNDER THE KIDNEY CAPSULE

Recipient mice were anaesthetized with avertin (section 2.17) and shaved on the left flank with electric animal clippers to remove hair from the incision site. Instruments were sterilized by boiling immediately prior to use. The left flank was swabbed with 70% ethanol and a vertical midline incision was made in the skin using a scalpel blade. The abdominal wall was cut with scissors and the kidney exposed and held in position with a cotton swab. A small incision was made in the kidney capsule using a 19 gauge needle (Terumo, Australia) and a space was made between the capsule and kidney parenchyma, using a rounded 27 gauge Luer lock needle. The transplant tissue was then inserted under the capsule using the modified Luer lock needle as a probe. During the procedure the kidney was swabbed regularly with HEPES-buffered HBSS to prevent dehydration. The kidney was then replaced in the abdominal cavity and the incised skin was sutured together with autoclips (9 mm; Clay Adams, USA). The transplanted mice were placed under an incandescent lamp until they recovered from anaesthesia.

TABLE 2.1

H-2 haplotypes of mouse strains used in this study

	K	I-A	I-E	D	Background strain
C57Bl/6J (B6)	b	b	b	b	type
BALB/c	d	d	d	d	type
CBA/H (CBA)	k	k	k	k	type
B10.AQR	q	k	k	d	C57Bl/10J
B10.A	k	k	k	d	C57Bl/10J
B10.T(6R)	k	q	q	d	C57Bl/10J

TABLE 2.2

Characteristics of the tumour cell lines used in this study

Name	Strain of origin	Cell type	Culture media
P815	DBA/2	Mastocytoma	DMEM + 10% HIFCS
EL4	B6	T cell lymphoma	DMEM + 10% HIFCS
R1(TL^+)	C58	T cell lymphoma	DMEM + 10% HIFCS
WEHI 3	BALB/c	Myelomonocyte	RPMI + 5% HIFCS
MLA 144	Gibbon	T cell lymphoma	RPMI + 5% HIFCS
K562	Human	Erythroid leukaemia	RPMI + 5% HIFCS

TABLE 2.3

Estimation of the reproducibility of IL3 determination using ^{3}H-thymidine incorporation

IL3 activity determined from replicate titrations on day:			
	Day 1	Day 2	Day 3
A	1.60	1.60	1.80
B	1.65	1.50	1.80
C	1.86	1.50	1.80
D	1.65	1.43	1.80
E	1.60	1.53	1.86
F	1.65	1.40	2.00
$\bar{X}$	1.67	1.50	1.84
S.D.	0.09	0.07	0.08

Analysis of variance table

Source of variation	Sum of squares	Degrees of freedom	Estimate of variance
Between days	0.3675	2	0.184
Within days	0.1052	15	0.007

TABLE 2.4

Estimation of the reproducibility of IL3 assays employing measurement of hexosaminidase levels

IL3 activity determined from replicate titrations on day:	Day 1	Day 2	Day 3
A	2.58	2.55	3.06
B	2.45	2.46	3.00
C	2.50	2.46	3.10
D	2.58	2.67	3.30
E	2.85	2.55	3.06
F	2.70	2.55	3.15
$\bar{X}$	2.61	2.54	3.12
S.D	0.15	0.08	0.10

Analysis of variance table

Source of variation	Sum of squares	Degrees of freedom	Estimate of variance
Between days	1.202	2	0.601
Within days	0.2792	15	0.0186

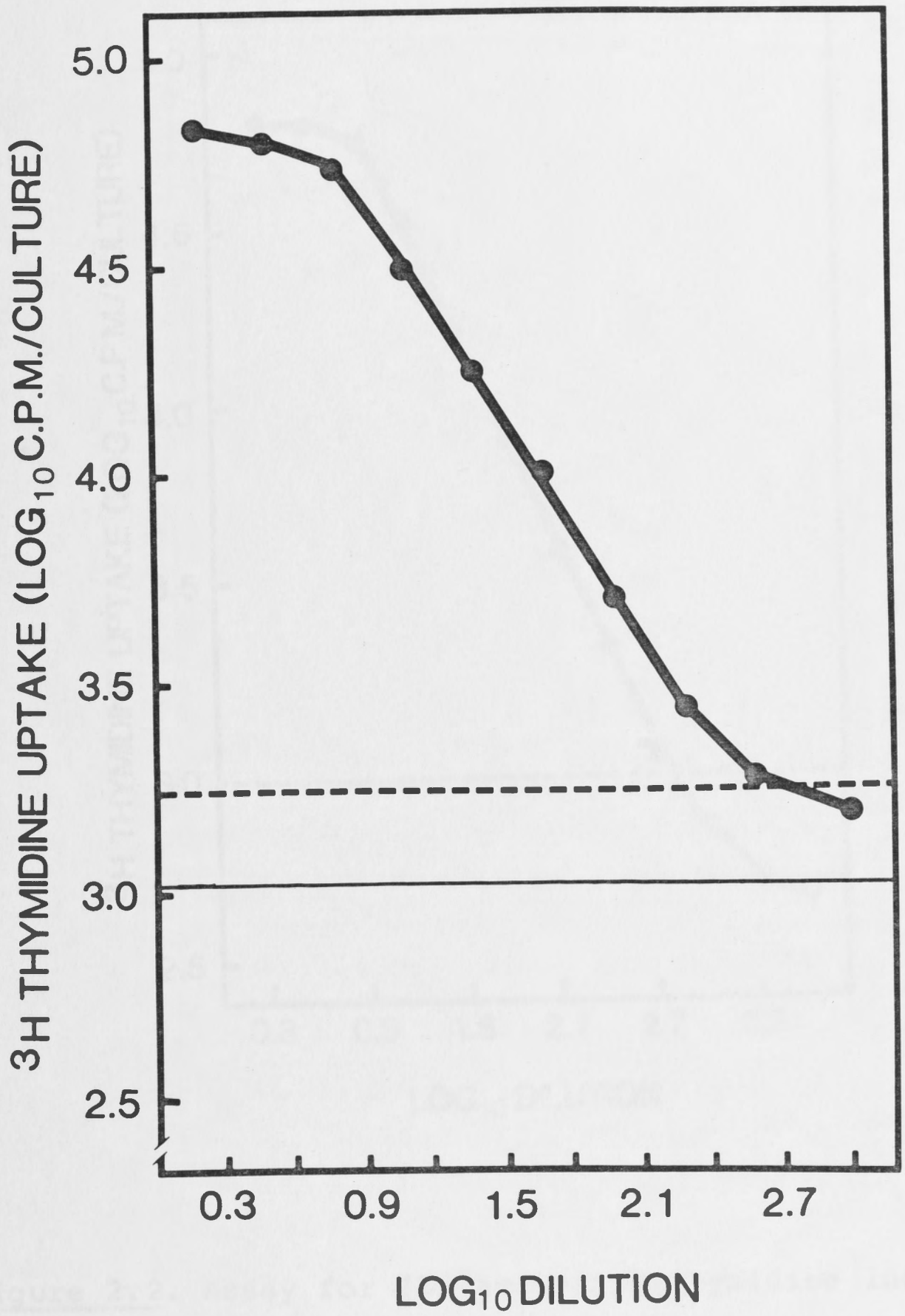

Figure 2.1. Assay for IL2 activity. CS was prepared as described in section 2.6 and titrated in the IL2 assay (section 2.10). Each point is the arithmetic mean of duplicate cultures. The thin line is the mean background incorporation determined in the absence of CS. The dotted line is the arbitrary endpoint level ($\bar{X}$ x 3.S.D.).

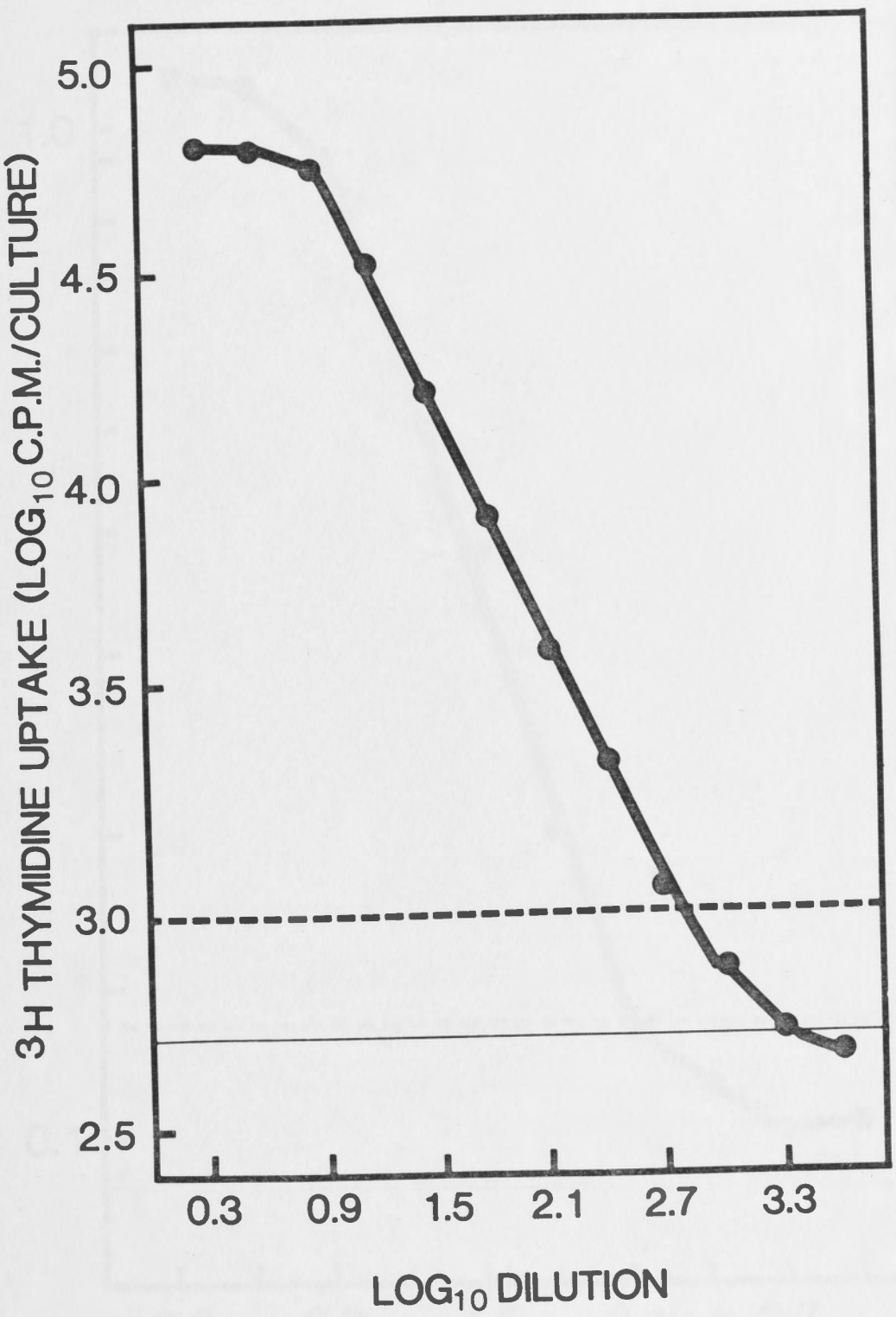

Figure 2.2. Assay for IL3 activity: Thymidine incorporation. A 1:10 dilution of a WEHI 3 tumour cell supernatant was titrated in the IL3 assay (section 2.12). Proliferation of the FD IL3 indicator cell was measured using ^{3}H thymidine incorporation (section 2.12B). Log_{10} c.p.m. is plotted against log_{10} reciprocal dilution of supernatant. Lines indicating the mean background incorporation in the absence of WEHI 3 supernatant and the arbitrary endpoint ($\bar{X}$ + 3 x S.D.) are shown.

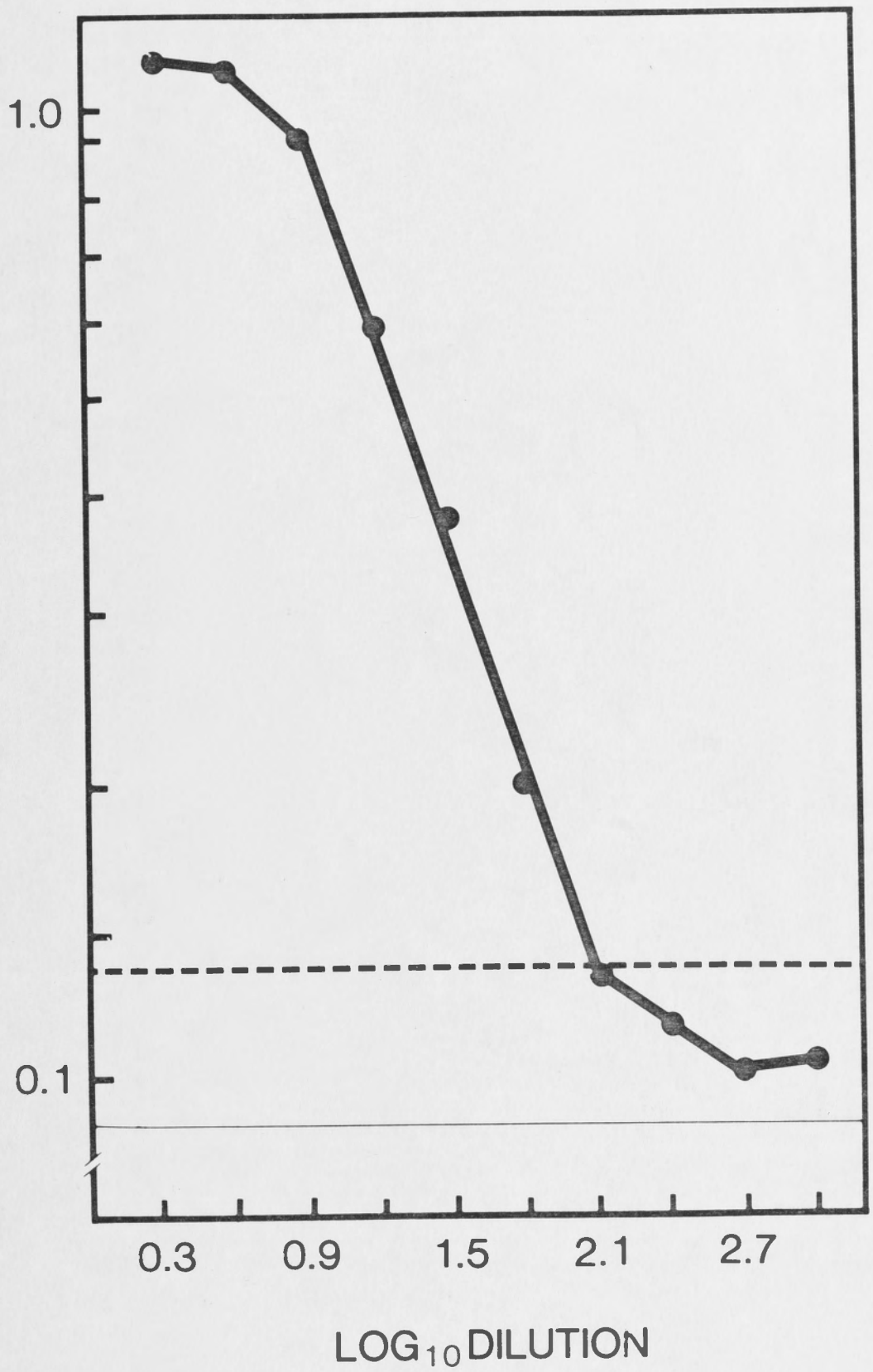

Figure 2.3. Assay for IL3 activity; Measurement of enzyme levels. A WEHI 3 supernatant was titrated in an IL3 assay using the FD cell line. Proliferation was measured by the hexosaminidase level per well (section 2.12B). Log_{10} O.D. is plotted against log_{10} reciprocal dilution of supernatant. Lines indicating the mean background incorporation in the absence of WEHI 3 supernatant and the arbitrary endpoint ($\bar{X}$ + 3 x S.D.) are shown.

CHAPTER 3

CHARACTERISTICS OF LYMPHOKINE RELEASE

3.1 INTRODUCTION

The release of biologically active molecules, generically termed "lymphokines" from T lymphocytes is an important feature in generating an immune response. Lymphokine activities have been described which affect macrophage migration and activation (Fowles et al, 1973; Bloom and Bennett, 1966), which are chemotactic for a number of cell types such as eosinophils (Cohen and Ward, 1971) and monocytes (Ward et al,1970) and which influence cell differentiation, most notably of B lymphocytes (Swain et al, 1981, Andersson et al, 1980). The release of one of these factors, the T cell growth factor or IL2, was the subject of a study by Andrus and Lafferty (1981). The major finding of this work was that activation of IL2-secreting cells required antigen presented on specialized stimulator cells in a way which is analagous to the activation of cytotoxic T cells. Once activated, however, T cells would initiate IL2 release following exposure to any cell bearing the appropriate antigen, including cells rendered metabolically inactive by UV-irradiation. This observation lead to the conclusion that an antigen signal only controlled IL2 release from activated T cells. Lafferty et al, (1983) have extrapolated from this to suggest that all lymphokines released from activated T cells are controlled solely by the antigen signal. If this is the case lymphokine release from activated T cells could serve as a simple means of studying the necessary physical characteristics of antigens in providing the antigen signal.

The purpose of the work described in this chapter was to examine the reproducibility of lymphokine release assays and test the assertion that lymphokines are under identical cellular control by comparing IL2 release to that of IL3. IL3 is a haemopoietic growth factor with physical characteristics distinct from IL2 (Ihle et al, 1983).

3.2 MATERIALS AND METHODS

3.2.1 Preparation of seven day-activated T cells

Seven day-activated T lymphocytes were prepared from a B6 anti-BALB/c MLR as described in section 2.9. Cells prepared in this manner are 99% Thy 1^+ (Woolnough and Lafferty, 1979).

3.2.2 Triggering of lymphokine activity from seven-day activated T cells

A) 3 ml assay: Seven day-activated T cells were suspended at 2×10^6/ml in EMEM containing 10^{-4}M 2-ME. P815 target cells were UV-irradiated at 960 $\mu W/cm^2$ for 4 mins and suspended in EMEM plus 10^{-4}M 2-ME at 8×10^6 cells/ml. An aliquot of 1.5 ml of each suspension was mixed in a Falcon 3002 culture tube (Falcon, California). These cultures were gassed with a 7% O_2; 10% CO_2; 83% N_2 for 6 hours at 37°C. At the end of culture each tube was centrifuged at 1000g for 10 min to sediment cells and cell debris. The supernatants were then decanted and stored at 4°C.

B) Micro-release assay: Cells used for this assay were prepared as described for the 3 ml release assay. To initiate the reaction 100 µl of T cell suspension was added to 100 µl of target cell preparation in individual wells of a 96 well microtitre tray (NUNC, Denmark). These plates were

then incubated at 37°C in a humidified atmosphere of 7% O_2; 10% CO_2; 83% N_2, for the appropriate time. At the end of culture 100 µl was removed from each well and transferred in two aliquots of 50 µl to the first wells of a new microtitre tray in preparation for a lymphokine assay. Prior to assay, 50 µl of media was added to each supernatant aliquot to take the final volume to 100 µl. Thus each lymphokine containing supernatant was assayed in duplicate.

3.2.3 Treatment of supernatants for inhibitor removal

To remove the IL2 inhibitor (Andrus and Lafferty, 1981), 2 of the 3 mls from one lymphokine release experiment were removed and diluted to 10 ml with fresh EMEM plus 10^{-4} M 2-ME. This diluted supernatant was then supplemented with 0.2 mls HIFCS and concentrated to less than 2 mls using a B15 Minicon concentrator fitted with a PM 10 ultrafiltration membrane (Amicon Corp, Lexington, Mass). The concentrated supernatant was removed and reconstituted with fresh medium to its initial volume of 2 ml. This supernatant was then sterilised by filtration through a 0.22 µm disposable filter (Millipore Corporation) and stored at -20°C until assayed.

3.2.4 Lymphokine assays

Estimates of IL2 and IL3 activity in cell supernatants

were determined according to the methods described in sections 2.10 and 2.12 respectively.

3.2.5 Preparation of mRNA from activated T cells

Total mRNA was prepared from an aliquot of 10^8 seven day activated T cells suspended in 0.1 ml serum-free EMEM using the method of Maniatis (1982). The RNA preparations were electrophoresed on a 2.2M formaldehyde-10% agarose gel (Lehrach et al, 1977) and transferred to nitrocellulose (Maniatis, 1982). The gel was prehybridized for 5 hours at 42°C in a buffer containing 50% formamide, 1 x Denhardts solution (Denhardt, 1966), 100 µg/ml salmon sperm DNA, 0.1% sodium dodecylsulphate and 5 x SSPE solution (SSPE = 0.15M NaCl, 10mM NaPhosphate, 1mM EDTA, pH 7.7). The nitrocellulose membranes were then hybridized for 20 hours at 42°C in the same buffer containing 6 x 10^6 c.p.m./ml of murine IL3 cDNA probe (Fung et al, 1984) labelled with ^{32}P-dATP by random priming (Taylor et al, 1976). Autoradiography was performed using an intensifying screen and preflashed film (Laskey, 1980).

3.3 RESULTS

3.3.1 The reproducibility of the 3ml lymphokine release assay

Stimulation of IL2 from activated T cells is effected equally by UV-irradiated and live target cells (Andrus and Lafferty, 1981). In this study, therefore, all target cells are UV-irradiated to eliminate any metabolic contribution from these cells. Andrus and Lafferty (1981) also described an inhibitory activity which was found in T cell supernatants and which could completely mask the presence of IL2. This inhibitor appeared to bind to Amicon PM 10 ultra filtration membranes, thus offering a simple means for its removal.

In developing an assay for IL2 release, 3 ml was the minimum supernatant volume which could satisfactorily be treated for inhibitor removal.

An experiment was performed to gain some estimate of the error involved in obtaining IL2 using 3 ml supernatant volumes and PM 10 treatment for removal of the inhibitor. Eight replicate cultures containing 1.5×10^6 seven-day activated T cells and 6×10^6 UV-irradiated P815 were prepared and cultured as described in section 3.2.2. The results of an IL2 assay of both untreated and treated (as described in section 3.2.3) supernatants are shown in Table 3.1.

Two points arise from this data. Firstly, there is no evidence of the IL2 inhibitor which can be present in supernatants prepared in this manner and, secondly, that the standard deviation of treated and untreated samples are similar, indicating that the bulk of the error inherent in this procedure lies in the IL2 assay and not in the preparation of each replicate. As the inhibitor did not appear to be produced, the above procedure was repeated on a further two days without the PM10 treatment. The data and an analysis of variance table are depicted in Table 3.2. Between day variation precludes the effective comparison of IL2 titres obtained on different days. Within days however, the assay is highly reproducible.

The expected standard error (S.E.) about the mean of 2 replicates would be 0.04 and on one replicate, 0.08. These figures yield 95% confidence intervals of 0.08 and 0.16 respectively.

3.3.3 Reproducibility of a micro-release assay

Many experiments require at least 3 ml of lymphokine supernatant, either for PM10 treatment or for assay of a number of different lymphokine activities. The estimation of dose response curves, relating antigen, T cell dose and lymphokine titre, requires only a single assay and can, therefore, be achieved with a supernatant volume of 200 µl.

The previous study showed no evidence of an IL2 inhibitor which was present in supernatants produced in a very similar way by Andrus and Lafferty (1981). The removal of this constraint allowed the assay volume to be reduced to 200 µl.

The reproducibility of a "micro-assay" was determined by repeating the procedure described in section 3.2.2B on four occasions over a four week period and employing eight replicates per day.

The results and an analysis of variance table are presented in Table 3.3. As with the 3 ml release assay, significant day to day variation in titre is present, most probably due to variation in the separate T cell preparations. Removing the between day variation reveals an extremely reproducible determination when taken on a single day. Duplicate samples taken on the same day would be expected to have a S.E. about the mean of 0.037 and a 95% confidence interval of 0.074.

The standard error expected on a single determination is no greater than that found for the IL2 assay itself (0.13, as assessed by Lafferty et al, 1980). Thus, the bulk of the sample variation occurs not in the release step but in the subsequent lymphokine assay.

3.3.4 The time course of IL2 and IL3 release are similar

The relationship between the time of release of IL2 and IL3 from activated T cells was examined. The 3 ml assay as described in section 3.2.2A was used. Supernatants were harvested at varying times up to 27 hours. Assays for IL2 and IL3 were performed on both untreated and PM10 treated supernatants. The 32D IL3 responsive cell was employed in the IL3 assay. The results are shown in Figure 3.1. T cells alone did not release detectable lymphokine at any time. IL2 activity rises to a peak at 8 hours before rapidly falling to undetectable levels. This apparent decrease in titre can largely be accounted for by an inhibitor, the presence of which masks the IL2 assay. Thus, when treated for inhibitor removal using the PM 10 method of Andrus and Lafferty (1981), a major portion of the maximum titre is restored. Following this treatment a further twofold decrease in titre between 10 and 27 hours is observed. This additional effect is probably due to the consumption of IL2 from the media by the T cells themselves. The ability of activated T cells to remove IL2 from culture supernatants has been described previously (Watson and Mochizuki, 1981). IL3 activity also reaches its peak titre at 8 hours and remains at this level until the completion of the experiment. Thus, the IL3 assay is not affected by the IL2 inhibitor, nor is IL3 activity absorbed from the cultures by the T cells. It is also worth noting that the IL3 titres

obtained are substantially greater than the IL2 titres obtained from the same supernatant. The IL3 assay is either more sensitive than the IL2 assay, or greater quantities of IL3 are produced by this cell population.

When plotted as a percentage of the maximum response a close parallel in the time course of IL2 and IL3 production over the first 10 hours is observed (Figure 3.2).

In Figure 3.3 a time course of IL3 production by cells taken from an MLR at 4 days is presented. The four-day cells release some IL3 in the absence of antigen stimulation although this level rises when antigen is supplied. This contrasts with the seven-day activated T cells which give no detectable release in the absence of antigen stimulation. This background release is probably due to the presence of cells which were triggered by antigen just prior to removal from the MLR, or to the residual presence of antigenic cells. This result emphasises the importance of the 3 day expansion step after the initial MLR. During this period the specifity for H-2 bearing target cells is maintained but the spontaneous background seen at 4 days is eliminated.

3.3.5 A comparison of 32D and FD IL3 responsive cell lines

The cell 32D was used to measure IL3 in the above section. However, the titration curves obtained from these

assays were not always linear when plotted as $\log_{10}$ cpm versus $\log_{10}$ supernatant dilution. An example of this is shown in Figure 3.4. Such non-linearity is never observed in IL2 assays and when it occurs in the 32D assay an accurate determination of the endpoint is difficult.

Another IL3 sensitive cell line, FD, became available and a comparison between it and 32D was performed. Table 3.4 indicates that 32D was consistently more sensitive than FD, yielding endpoint titres higher by 0.2 to 0.65 $\log_{10}$ units. However, the consistency of both the slope and the linear section of the curve obtained from the FD assay was generally superior to 32D. For these reasons FD was adopted as a standard IL3 indicator cell.

The standard error of the FD assay as determined in section 2.11A is 0.07. This is similar to the IL2 assay. As this error represents the major part of the error associated with the lymphokine release procedure, it can be used to estimate the error of IL3 determinations in lymphokine release assays.

3.3.6 Measurement of antigen dose responses: IL2 versus IL3

Figure 3.5 shows the result of performing a micro-

release assay holding the number of activated T cells constant at 10^5 cells/well and varying the number of P815 targets through 2-fold dilutions from 10^3 to 2×10^6/well. Four replicate supernatants per dilution were generated, two being used for an IL2 assay and two for an IL3 assay. Clearly, there is a very close relationship between the amount of IL2 and IL3 produced in response to changes in antigen dose. A more detailed analysis of this dose response curve is given in chapter 7.

3.3.7 The T cell dose response curve

The result of measuring the levels of IL2 and IL3 produced in cultures where the number of antigenic cells is held constant and the number of T cells is varied in 2-fold steps from 10^3 to $10^{5.7}$ is shown in Figure 3.6. Both IL2 and IL3 determinations lie on a straight line for most of the T cell doses used. The gradients of these two lines are not significantly different ($p < 0.05$); for IL2 the slope and 95% confidence interval is 1.03 ± 0.1, and for IL3, 1.16 ± 0.06. The closeness of this gradient to 1.0 suggests an empirical relationship of the form;

$$\log_{10}(LK) = \log_{10} N_T + \log_{10} k \qquad (3.1)$$ or,

$$(LK) = kN_T \qquad (3.2)$$

where (LK) is the lymphokine titre, N_T is the number of T

cells/well and k is a constant related to the average titre attributable to one lymphokine releasing cell over the period of assay.

3.3.8 The antigen dose response curve using live and UV-irradiated P815 as target cells

The antigen dose response relationships between IL3 titre and target cell dose, for both live and UV-irradiated target cells are shown in Figure 3.7. The two curves are similar, the most notable distinction being a sharp decrease in IL3 production when live P815 were added at $10^{6 \cdot 0}$/well. This decrease may have been caused by the depletion of culture media components by such a large number of metabolically active tumour cells.

3.3.9 Stimulation by mitogen involves synthesis of new lymphokine-encoding mRNA

An aliquot of 10^8 seven day-activated T cells was incubated with or without 5 µg/ml Con A for 2 hours in 50 ml EMEM containing 10^{-4}M 2-ME. The cells were then washed and recultured with fresh medium for a further 4 hours.
The method for detection of IL3 encoding mRNA in these cell preparations is described in section 3.2.5.

Con A was used to trigger lymphokine production in these experiments to eliminate the problem of increased total mRNA which would be created by using P815 target cells.

Figure 3.8 shows that IL3 encoding mRNA was undetectable in unstimulated cells but could be measured in cells exposed to Con A. Thus, triggering of IL3 release requires transcription of specific mRNA.

3.4 DISCUSSION

The measurement of IL2 release from activated T cells can be used to indicate exposure of these cells to an appropriate antigenic source (Andrus & Lafferty,1981). Data presented in sections 3.3.1 and 3.3.2 demonstrates that the quantity of IL2 released in replicates is extremely reproducible and that the bulk of the variation observed can be attributed to the IL2 assay itself. The release of IL3, like IL2 follows exposure of activated T cells to an antigenic target cell. The assay of this lymphokine has an advantage over IL2 measurement, in that it is not affected by an inhibitor, produced in culture, which can completely mask IL2 activity. In this study the IL2 inhibitory activity was detected in T cell supernatants after 20 hours of culture. However, a similar study by Andrus and Lafferty (1981) found that IL2 activity was inhibited at any time following antigen exposure.

The existence of this inhibitor may cast some light upon a dispute concerning the release of IL2 from class 1 MHC-reactive T cells. Studies by Andrus et al (1981) and Swain (1981), have reported IL2 production from class 1 MHC-reactive T cells. Other reports describe IL2 release by T cells carrying the lyt 2 marker (Guerne et al, 1984; Kern et al, 1981). This cell surface antigen is a phenotypic characteristic of class 1 MHC-reactive T cells (Swain, 1983). In contrast, work by Miller and Stutman (1982) and Kelso and MacDonald (1982) has failed to find evidence for IL2 release from lyt 2+ T cells.

In the latter study, the number of T cell precursors which gave rise to IL2 secreting T cell clones was found to be one fiftieth of that found for lyt 2^- T cells.

In a more recent report, Kelso et al (1984), divides class 1 MHC-reactive T cells into cytolytic and non cytolytic, and the cytolytic group into IL2 dependent and IL2 independent groups. IL2 releasing cells were never observed in the IL2 dependent cytolytic group but were found amongst the other subdivisions. It is interesting to note that these authors found IL2 production to be a variable property of an individual clone whereas the rates of release of other activities, such as MAF or CSF were very stable. Also IL2 was often observed at 24 hours but had disappeared by 48 hours, a phenomenon attributed by these authors to consumption by the T cells.

A possible alternative explanation of these findings is that in a number of clones an inhibitor of IL2 activity is produced at various times following initiation of culture. This would account for the variability associated with IL2 detection, and for the apparent disappearance of IL2 with time. It would also explain why Andrus et al (1981) who routinely treated supernatants to remove the inhibitor, consistently found IL2 released in equivalent amounts by lyt 2^+ and lyt 2^- T cell populations. That this inhibitor exists and that it can affect the time course of IL2 production in the manner described by Kelso et al (1984) is demonstrated in Figure 3.2.

Although this explanation implies lyt 2^+ cells preferentially release this inhibitor, a hypothesis for which there is no evidence, it would nevertheless appear to be good practise to anticipate the presence of this inhibitor when testing for IL2 production.

The time course, antigen dose response curves and T cell dose response curves for IL2 and IL3 production following antigen triggering are similar. These observations suggest that in fact these two physically distinct lymhokines are under the control of the same cellular mechanism. In section 3.3.9 it was found that triggering of IL3 production involves transcription of new mRNA. If, as is suggested above, IL2 and IL3 are under similar control, then the release of IL2 should also require *de novo* synthesis of IL2 encoding mRNA. A study by Granelli-Piperno *et al*. (1984) of IL2 secreting Con A activated T cells found this to be the case.

It is not possible, without knowledge of the rates of IL2 and IL3 production from a single cell, to determine whether both lymphokines are produced within the same cell. However, numerous studies of cloned T cells have demonstrated that multiple lymhokines can be produced by a single T cell clone (Kelso and Glasebrook, 1984; Guerne *et al*, 1984; Wilde *et al*, 1984). It seems reasonable to conclude that at least a proportion, if not all, cells in the seven day-activated T cell population release both IL2

and IL3 following antigen exposure. However, even if produced in different cells, the above observations suggest that the same triggering requirements hold for all IL2, IL3 and IL2/IL3 releasing cells.

The addition of antigenic cells was used to initiate the lymphokine release reaction. It is not clear whether antigen is the sole requirement in triggering lymphokine from activated T cells. It has been reported that the tumour promoter PMA can enhance the lymphokine release capacity of activated T cells suggesting the possibility that more than one signal is involved (Wiscocil et al., 1985). In this study the possibility of the target cell producing a factor, such as IL1, which could modify the T cell response is eliminated by the routine UV-irradiation of any cells used as a source of antigen. In fact this treatment has little effect upon the antigen dose response curve (Figure 3.7).

It is also possible that the T cells themselves produce some factors which might enhance their own rate of lymphokine release. This is reportedly true of γ-interferon production, which according to Kelso et al. (1984) is enhanced and prolonged in the presence of IL2. This cannot be true however for the release of IL2 and IL3 described in this study, as the T cell dose response curve indicates a simple linear relationship between T cell dose and lymphokine titre. Any arrangement whereby T cells produce a

modifying factor would complicate the T cell dose response by placing an additional constraint upon the number of T cells. Thus, the rate of lymphokine release would depend upon both the number of T cells and the concentration of modifying factor. The latter variable is itself dependent upon the number of T cells, giving rise to a higher order relationship between T cell number and lymphokine titre. The difference between this conclusion and the results of Kelso et al (1984) may be due to our relatively short lymphokine release period (6 hours versus 24 hours) or that IL3 synthesis, unlike γ-interferon, is not affected by the IL2 concentration.

From the above discussion a simple model of lymphokine release emerges. A single signal initiated at the cell surface by either antigen or mitogen binding, mobilizes a range of lymphokine encoding mRNA. In Figure 3.9 this model is divided into four stages; antigen binding, transmission of the antigen signal, transcription of lymphokine encoding mRNA and translation and secretion of the active molecule. This model is used in the next chapter to analyse the site of action of CsA. In later chapters the model is important in a more detailed analysis of antigen and T cell dose response curves.

TABLE 3.1

Estimation of the error involved in the 3 ml lymphokine release assay following treatment for IL2-inhibitor removal.

Sample	IL2 activity (log_{10} titration endpoint)	
	untreated	PM-10 membrane treated
A	2.00	1.65
B	2.10	1.80
C	1.95	1.80
D	2.05	1.80
E	1.95	1.70
F	2.10	1.95
G	2.20	1.80
H	1.95	1.80
$\bar{X}$	2.04	1.79
S.D.	0.09	0.088

Cultures contained 1.5 x 10^6 seven day-activated T cells plus 6 x 10^6 UV-irradiated P815 in 3 ml EMEM. Each supernatant was harvested after 6 hours incubation at 37°C. PM10 treatment for inhibitor removal is described in section 3.2.3.

TABLE 3.2

The reproducibility of the 3 ml lymphokine release assay

	IL2 activity (log_{10} titration endpoint)		
	Day 1	Day 2	Day 3
A	2.00	1.85	1.35
B	2.10	1.95	1.40
C	1.95	1.95	1.60
D	2.05	1.95	1.50
E	1.95	1.90	1.65
F	2.10	1.90	1.50
G	2.20	1.90	1.60
H	2.10	1.90	1.50
$\bar{X}$	2.04	1.91	1.51
S.D.	0.09	0.035	0.103

Analysis of variance table

Source of variation	Sums of squares	Degrees of Freedom	Estimate of variance
Between days	1.3	2	0.65
Within days	0.135	21	0.006

TABLE 3.3

Reproducibility of the micro-release assay

IL2 activity (log_{10} titration endpoint)			
Day 1	Day 2	Day 3	Day 4
2.92	2.55	2.88	2.85
2.97	2.56	2.76	2.77
2.88	2.61	2.74	2.73
2.94	2.55	2.73	2.73
2.82	2.49	2.80	2.71
2.92	2.47	2.75	2.73
3.01	2.51	2.82	2.73
2.89	2.58	2.85	2.73
$\bar{X}$ 2.92	2.5	2.79	2.74
S.D. 0.06	0.05	0.055	0.045

Analysis of variance table

Source of variation	Sums of squares	Degrees of Freedom	Estimation of variance
Between days	3	0.6045	0.2015
Within days	28	0.0766	0.0027

TABLE 3.4

A comparison of 32D and FD as IL3 indicator cells

Test supernatant	32D Titre[a]	32D Slope[b]	FD Titre	FD Slope
WEHI 3 (1:10)[c]				
Batch 1	3.45	0.77	2.90	0.77
Batch 2	2.90	0.75	2.70	0.74
Con A stimulated T cell supernatant[d]				
Sample 1	3.0	0.5	2.60	0.77
Sample 2	3.4	0.5-0.7[e]	2.90	0.82

[a] Log_{10} endpoint titre.

[b] Slope of linear section of plot of log_{10} c.p.m. versus log_{10} supernatant dilution.

[c] 1:10 dilution of 24 hour supernatant from WEHI 3 culture.

[d] Seven day-activated T cells were incubated with 5 µg/ml Con A for 2 hours in 3 ml EMEM plus 10^{-4}M 2-ME. The cells were then washed and 3 ml fresh medium added. The cultures were then incubated a further 6 hours.

[e] Slope of this line appeared to have 2 straight sections one of slope 0.5 and the other of 0.7 (see Figure 3.4).

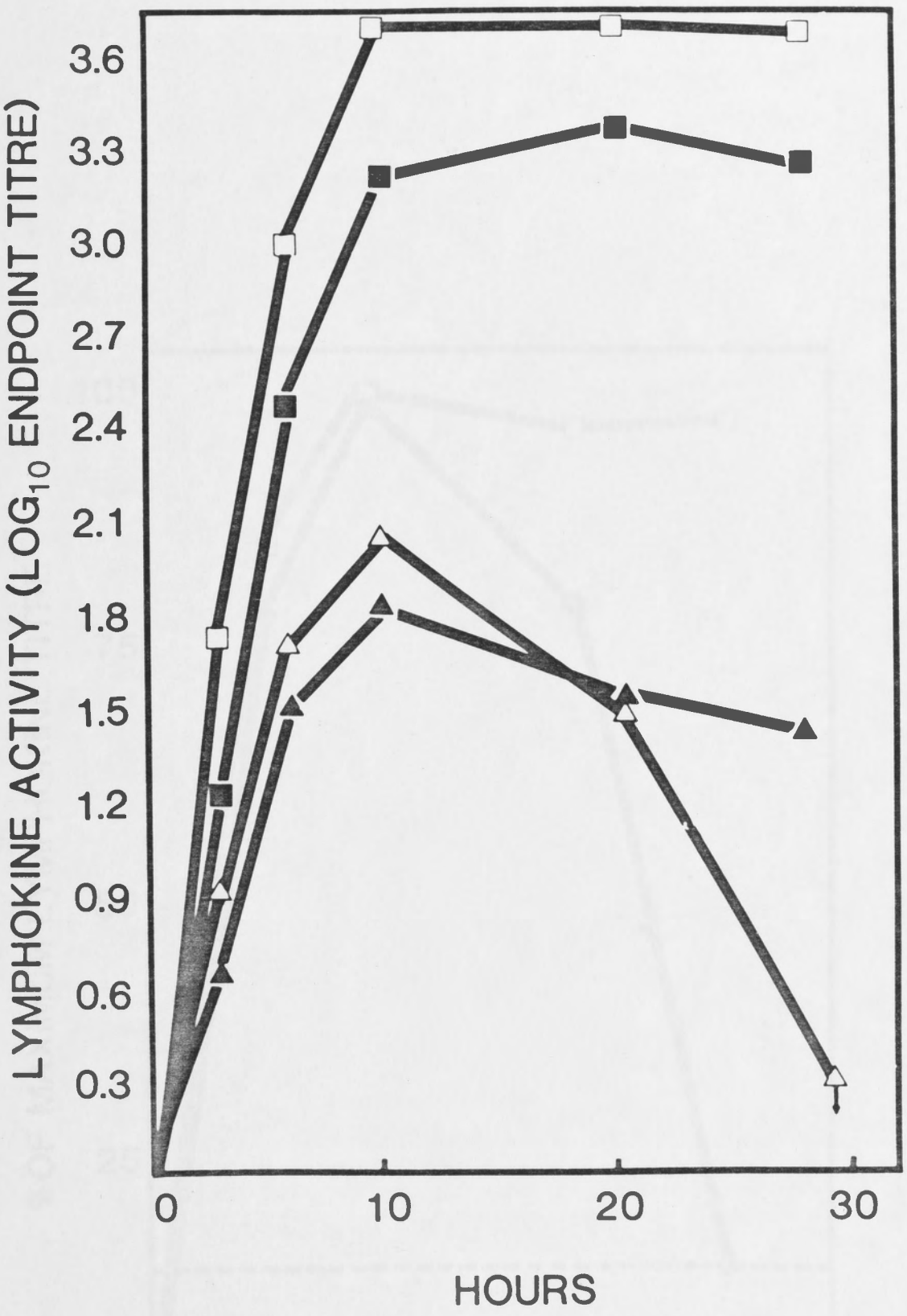

Figure 3.1. Time course of IL2 and IL3 production from seven day-activated T cells. 1.5 x 10^6 T cells were stimulated with 6 x 10^6 UV-irradiated P815 tumour cells in the 3 ml stimulation procedure described in section 3.2.2. Triangles represent the IL2 titre obtained before (open) and after (closed) treatment for inhibitor removal. The open and closed squares represent untreated and treated IL3 determinations. T cells incubated alone produced no detectable IL2 or IL3 at any time. Each point is the arithmetic mean of two cultures. Expected 95% confidence intervals are; for IL2, ± 0.08, and for IL3, ± 0.14 log units.

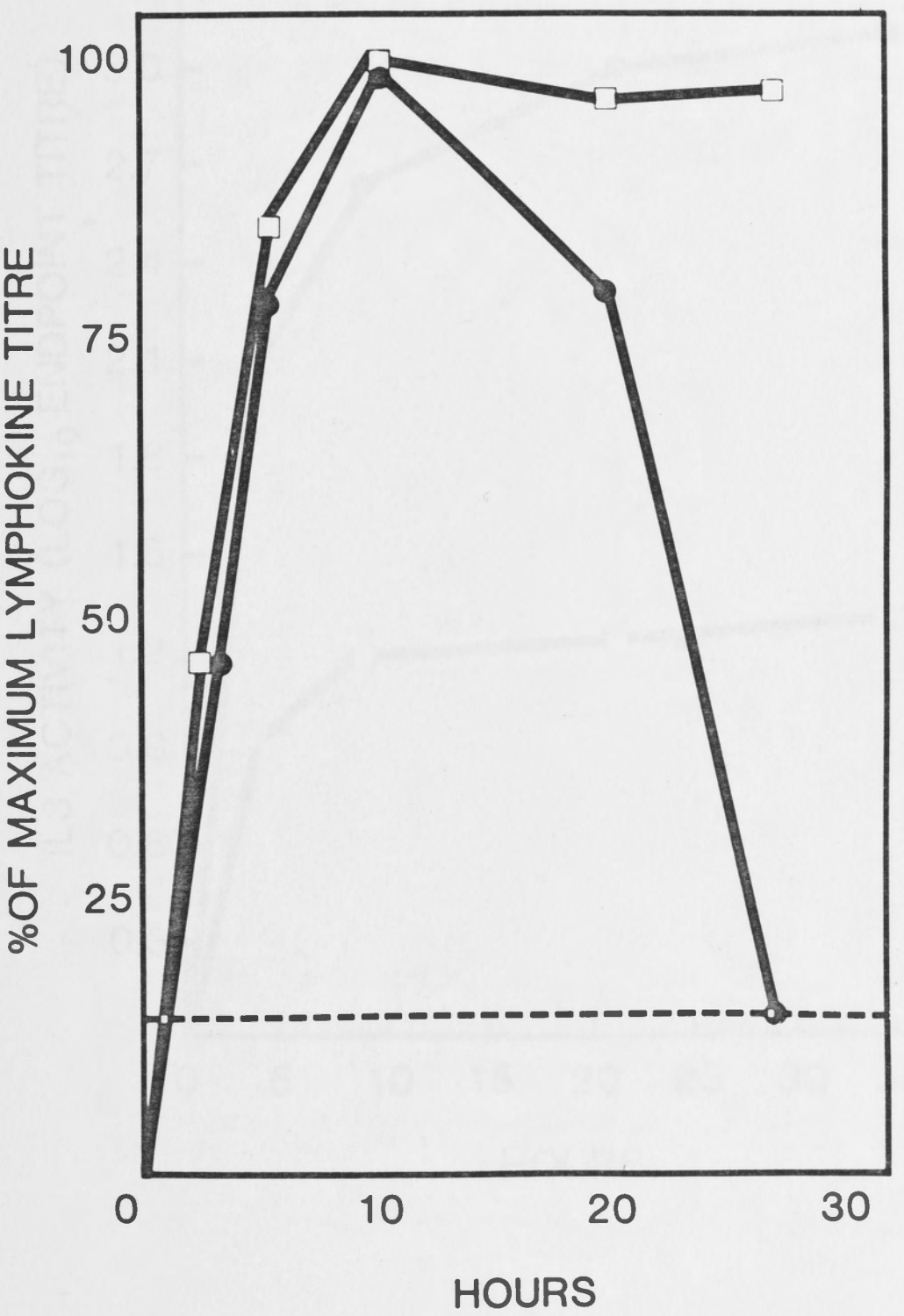

Figure 3.2. Time course of IL2 and IL3 production as a percentage of the maximum titre. In this figure the untreated supernatant IL2 (●) and IL3 (□) activities from Figure 3.1 are plotted as the percentage of the maximum titre.

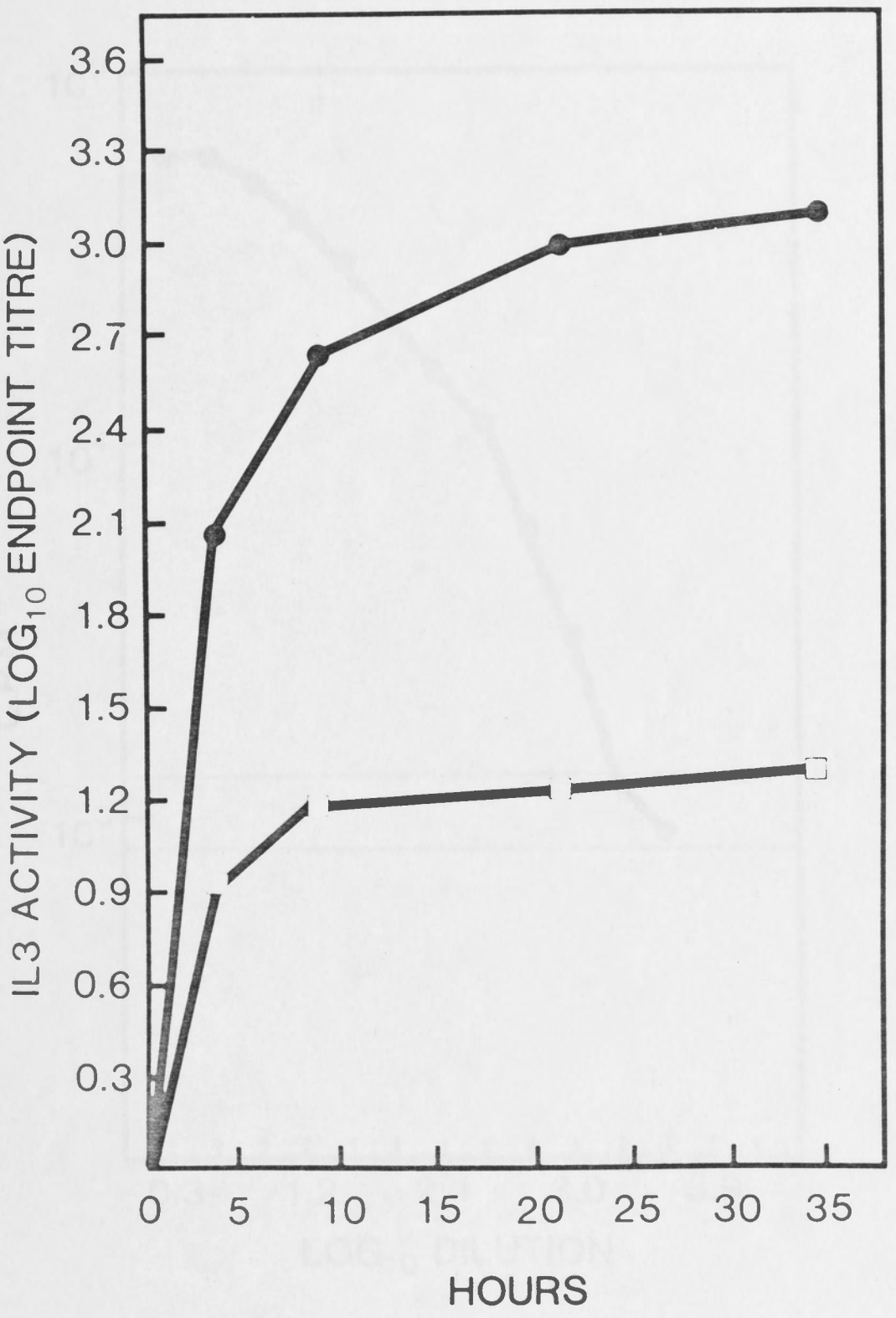

Figure 3.3. Time course of IL3 production from cells taken at day 4 of a B6 anti-BALB/c MLR. 1.5 x 10^6 cells were incubated either alone (□) or with 6 x 10^6 UV-irradiated P815 cell (●) for 6 hours according to the method described in section 3.2.2A.

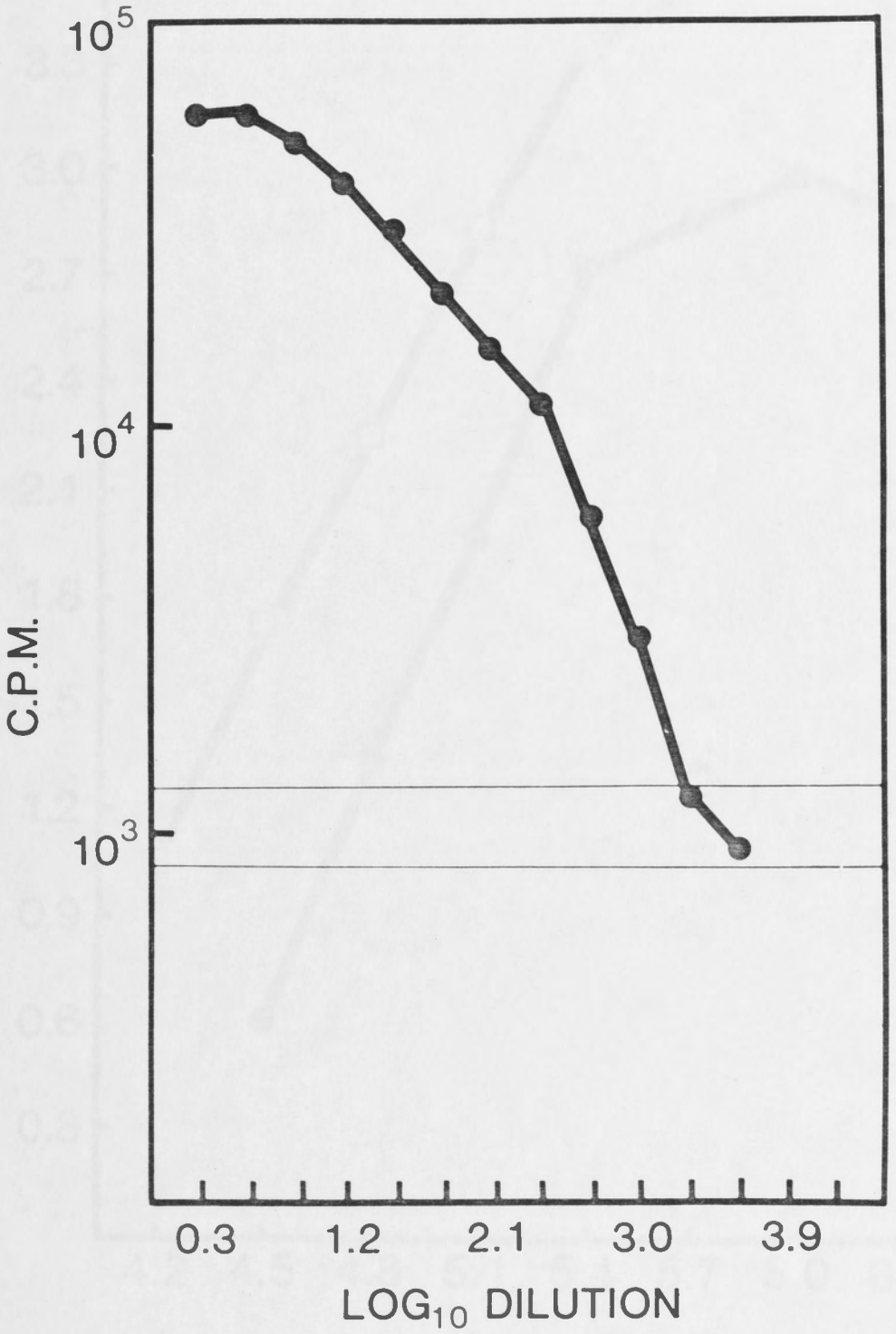

Figure 3.4. An example of a non linear dose response curve obtained with the 32D IL3 indicator cell line. The assay was performed as described in section 2.12A. Each point is the arithmetic mean of duplicate cultures. The titrated IL3 containing supernatant was obtained from Con A stimulated seven day-activated B6 anti-BALB/c T cells.

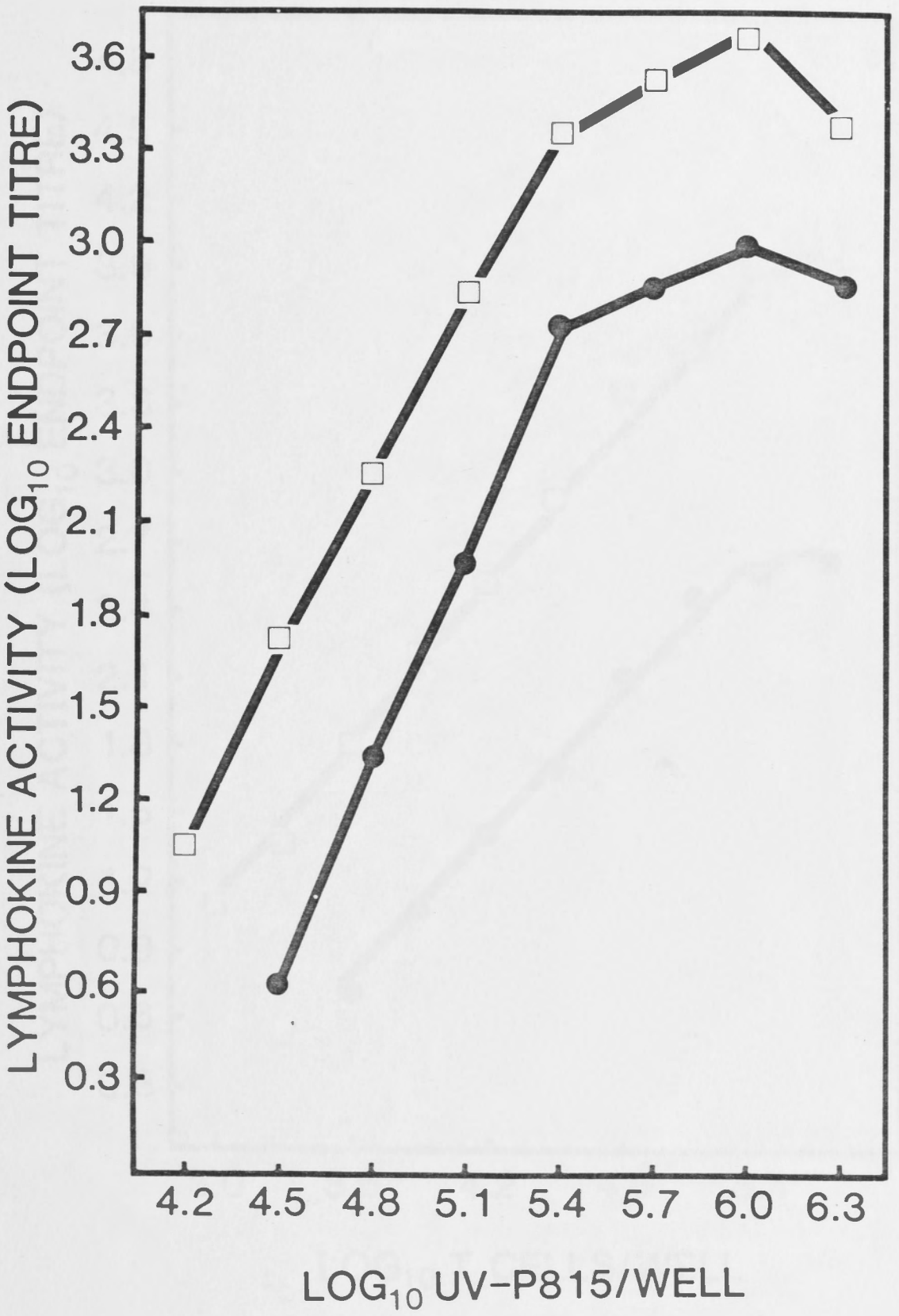

Figure 3.5. Antigen dose response. Varying numbers of UV-irradiated P815 were incubated with 10^5 B6 anti-BALB/c activated T cells for 6 hours in a final volume of 200 µl. Supernatants were assayed for IL2 (●) and IL3 (□) activity. The estimated 95% confidence interval of each point is; for IL2 ± 0.08 and for IL3 ± 0.14 $\log_{10}$ units.

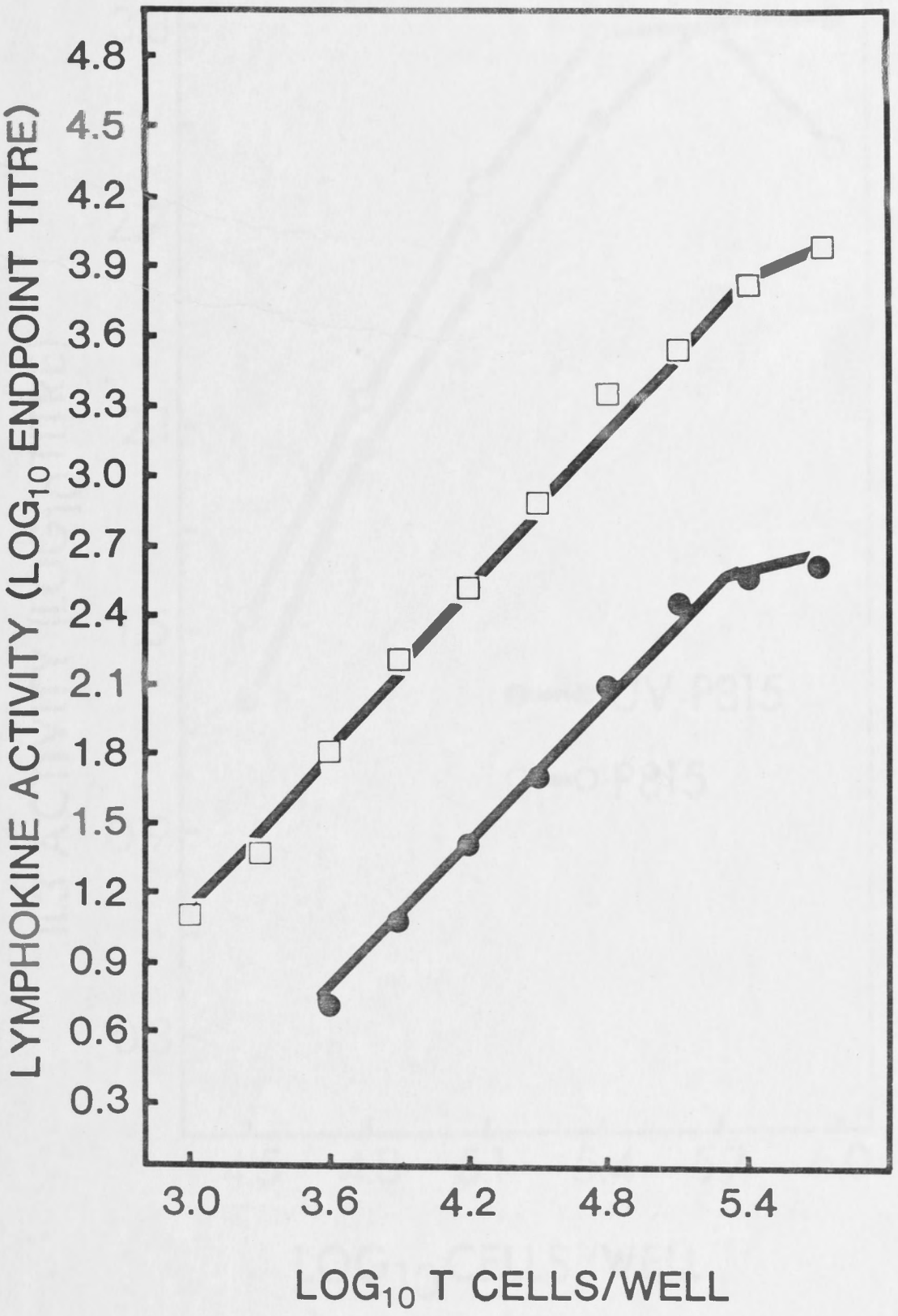

Figure 3.6. The T cell dose response curve for lymphokine release. Varying numbers of seven day activated T cells were incubated with 10^5 UV-irradiated P815 tumour cells in a final volume of 200 µl (section 3.2.2B). Supernatants were assayed for IL2 (●) and for IL3 (□). Estimated 95% confidence interval for IL2 determinations is ± 0.08, and for IL3, ± 0.14 $\log_{10}$ units.

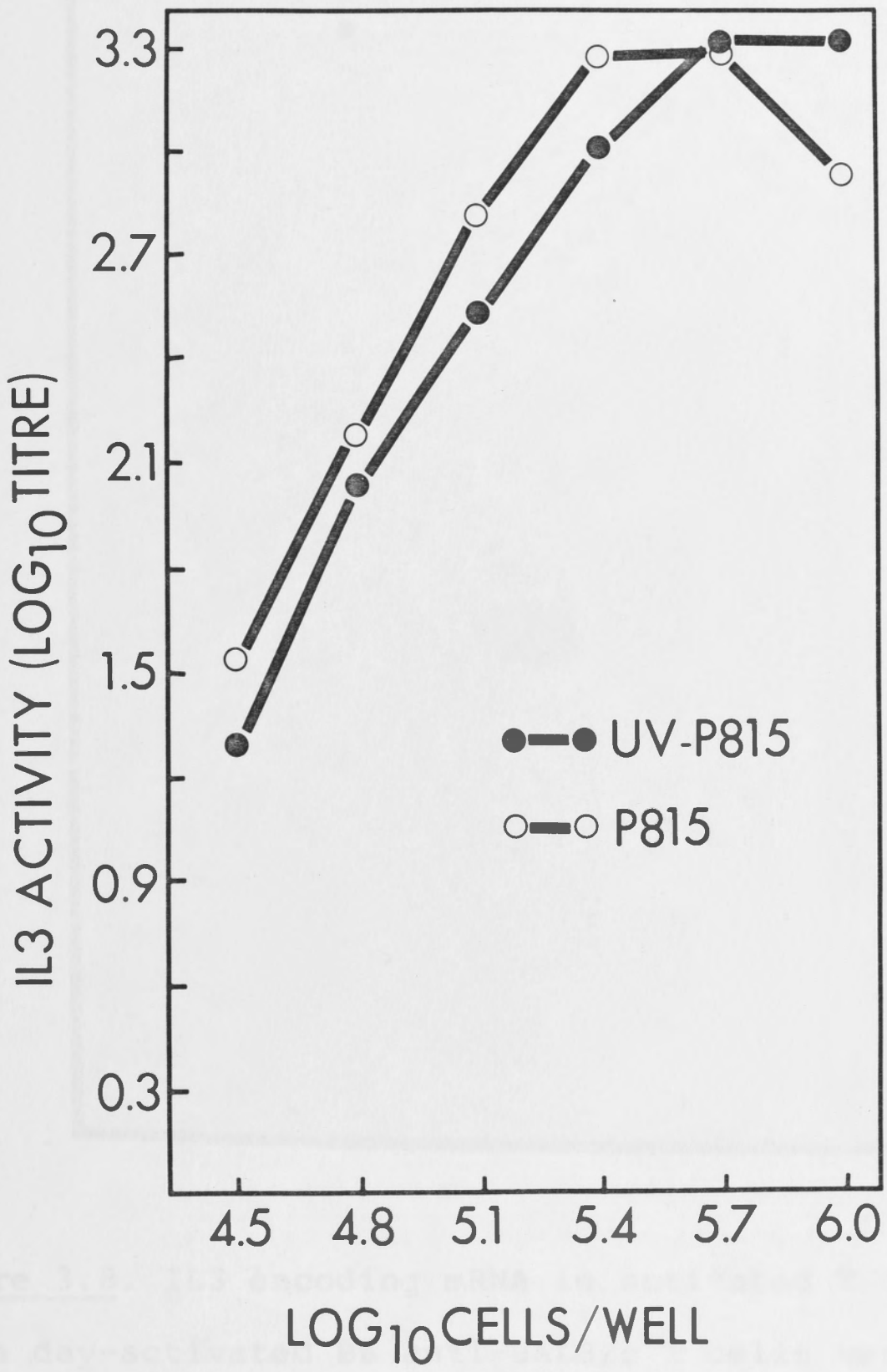

Figure 3.7. The P815 dose response curve for IL3 release using both live and UV-irradiated targets. Varying numbers of live or UV-irradiated P815 were incubated with 10^5 seven day-activated B6 anti-BALB/c T cells for 6 hours in the lymphokine release assay described in section 3.2.2B. Each supernatant was assayed for IL3 activity. The estimated 95% confidence interval for each point is ± 0.14 $\log_{10}$ units.

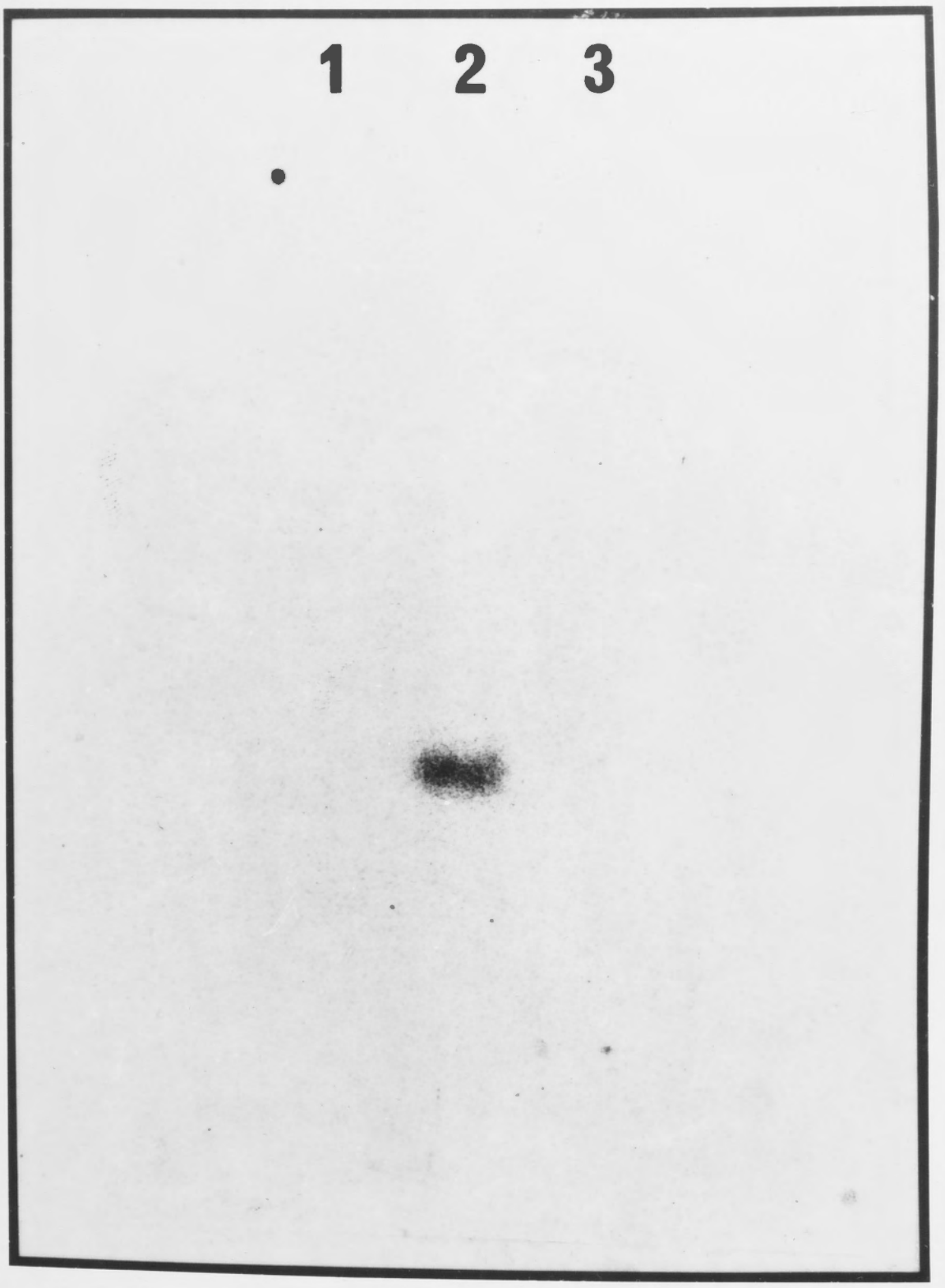

Figure 3.8. IL3 encoding mRNA in activated T cells. 10^8 seven day-activated B6 anti-BALB/c T cells were incubated alone (lane 1) or with 5 μg/ml Con A (lane 2) for 2 hours at 37^{o}C in EMEM plus 10^{-4}M 2-ME. These cultures were then washed with fresh medium and reincubated for 4 hours. At the end of this culture total mRNA was prepared and electrophoresed on formaldehyde-agarose gels, blotted to nitrocellulose and hybridized with a murine IL3 cDNA probe to detect IL3 encoding mRNA in the manner described in section 3.2.5.

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* This figure is also included in chapter 4 where lane 3 is relevant. The figure is included here as the first two lanes are important to this chapter.

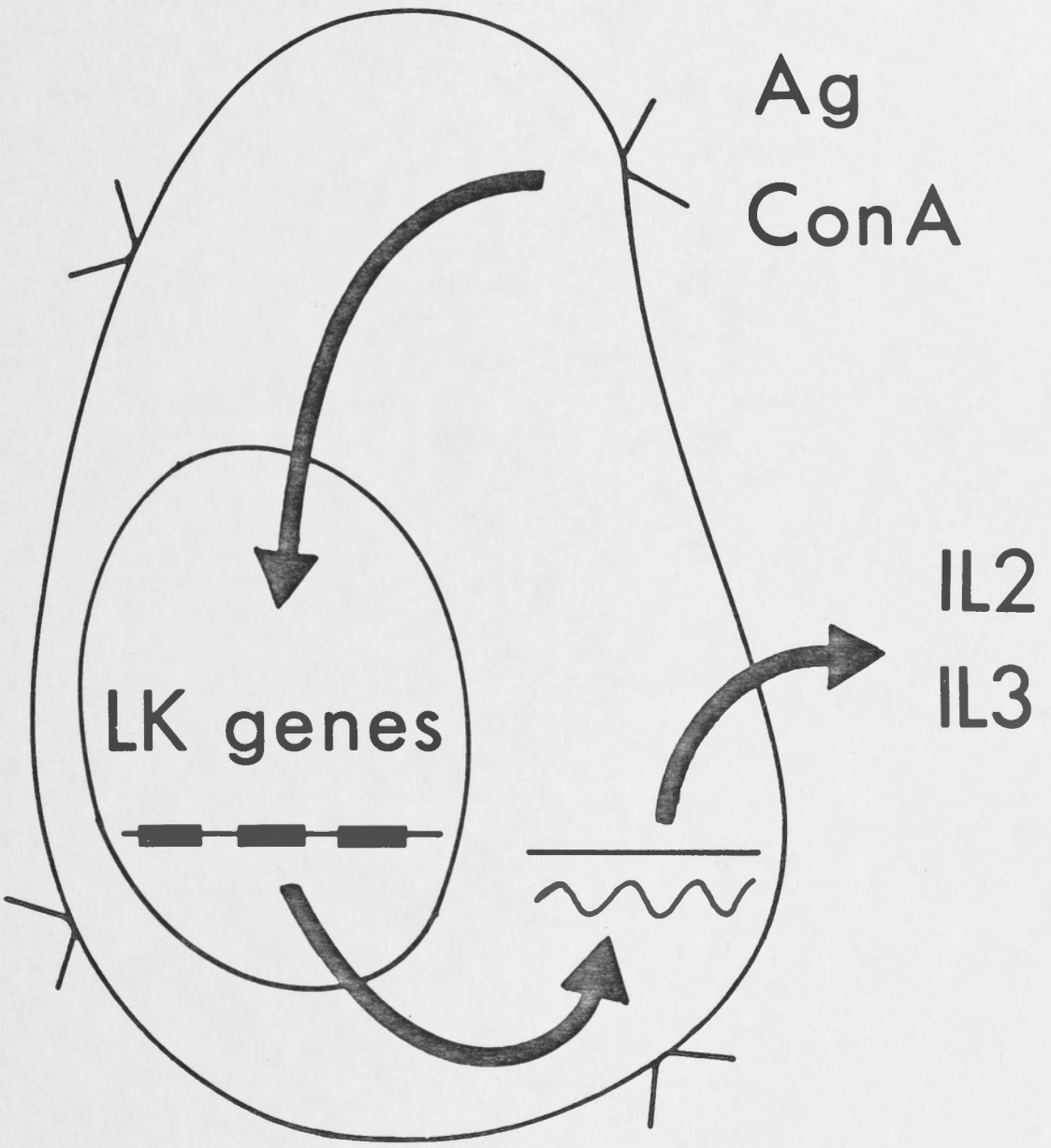


Figure 3.9. Model of lymphokine release from activated T cells depicting four steps in triggering lymphokine release; antigen (or mitogen) binding, transmission of the antigen signal, transcription of lymphokine encoding mRNA and translation and release.

CHAPTER 4

THE SITE OF ACTION OF CYCLOSPORINE

4.1 INTRODUCTION

Cyclosporine (CsA) is the first of a new generation of immunosuppressive agents with a specific site of action within the immune system (Borel, 1981). The agent is primarily lymphocyte specific and acts at an early phase of the cell's activation. A very low degree of myelotoxicity has allowed its use in clinical bone marrow transplantation. CsA appears to suppress lymphocyte function without damaging the activity of the reticuloendothelial system: animals treated with CsA show a normal capacity to clear colloidal particles or heterologous erythrocytes from the circulation (McIntosh and Thomson, 1980), and stem cell granulopoiesis is unimpaired (Hellmann and Goldman, 1980). Thus CsA provides a means of regulating the lymphocytic response to a tissue graft without completely laying waste the antibacterial defenses of the recipient. A greater understanding of the drug's site of action would be beneficial because of its clinical application and its potentially valuable use as a tool for dissecting the immune response.

Studies examining the effects of CsA in vitro have revealed an inhibitory effect on T cell activation, and lymphokine release, while having little or no effect on IL2-mediated T cell proliferation or the cytotoxic activity of activated T cells. This pattern of effects led Andrus and Lafferty (1982) to suggest that CsA blocks T cell behaviour by interfering with the antigen signal. The model of lymphokine release established in the previous chapter is used here to test this proposition.

4.2 MATERIALS AND METHODS

4.2.1 Activation of lymphocytes by H-2 alloantigens

Mixed lymphocyte cultures were prepared as described in section 2.8. Three mouse strain combinations were used: B6 anti-BALB/c, which represents a whole MHC haplotype disparity, B10.AQR anti-B10.A, a reaction against the K^k alloantigen and B10.AQR anti-B10.T(6R) an anti-I^q reaction.

4.2.2 Expanded populations of alloantigen activated T cells

Seven day activated T lymphoctyes were prepared from the above MLCs using the method described in section 2.9.

4.2.3 Triggering of lymphokine activity from seven-day activated T cells

A) Antigen stimulus. Activated T cells from CS-expanded populations were harvested, washed, and suspended at 2 x 10^6/ml in EMEM plus 10^{-4}M 2-ME. Cells to be used as a source of antigen were UV irradiated (section 2.7) for 4 mins and suspended in EMEM plus 2-ME at 8 x 10^{-6} cell/ml. Equal 1.5 ml volumes of target and effector cell suspensions were combined in a 5 ml plastic culture tube (Falcon, 2003) and

gassed with a 7% O_2: 10% CO_2: 83% N_2 gas mixture. These cultures were incubated at 37°C for 5 hours. At the end of culture, the tubes were spun at 700g for 10 minutes and the supernatant removed and stored at -20°C.

B) Con A stimulus. Activated T cells were suspended at 10^6 cells/ml in EMEM with 10^{-4}M 2-ME and 5 µg/ml Con A. An aliquot of 3 ml of this suspension was placed in a 5 ml culture tube (Falcon, 2003) and incubated at 37°C for 2 hours. The cultures were then spun at 200g for 5 mins, washed once, and refed with 3 ml of fresh medium. The cultures were then reincubated for 5 hours. The supernatant was harvested as described for antigen triggering (section 4.2.3A).

4.2.4 Lymphokine assays

Estimates of the IL2 and IL3 activity in cell supernatants were determined according to the methods described in section 2.10 and 2.12B respectively. All IL3 assays employed the FD indicator cell and were performed in duplicate.

4.2.5 Preparation storage and use of CsA

CsA was prepared by Sandoz Ltd. (Basel, Switzerland) and was a gift from Dr. Jean Borel of that company. As CsA is water insoluble a stock solution of 1 mg/ml was prepared in

dimethylsulphoxide (DMSO) and stored at -20°C until required. The stock solution was added directly to cell cultures or culture medium, usually diluted 1/1000 to provide a final concentration of 1 µg/ml. This procedure maintains CsA in a soluble form.

4.2.6 Preparation and detection of IL3 encoding mRNA from activated T cells

The procedure for detecting IL3-encoding mRNA in activated T cells has been described (section 3.2.5).

4.3 RESULTS

4.3.1 The effect of CsA on IL3 assays

The influence of CsA on the IL3 assay was tested by adding CsA at four different concentrations to a standard preparation of IL3 and estimating the IL3 content using the assay described in section 4.2.4. The results are shown in Table 4.1. Neither CsA concentrations as high as 3 µg/ml nor the solvent DMSO alone had any effect on the determination of the IL3 titre. It has been established that CsA has no inhibitory effect on IL2 assays (Andrus and Lafferty, 1982; Larsson, 1980; Bunjes et al, 1981; DosReis and Shevach, 1982). For these reasons no attempt was made to remove DMSO or CsA from cell supernatants required for lymphokine assays.

4.3.2 CsA blocks IL2 and IL3 release from antigen and Con A triggered, activated T cells

In Chapter 3 it was shown that antigen could be used to induce the release of the lymphokines IL2 and IL3. In this experiment CsA was added to the lymphokine release procedure at a final concentration of 1 µg/ml to test its effects on this reaction.

Antigen and Con A induced IL2 and IL3 release was achieved following the procedure described in section 4.2.3,

using, as the source of seven day-activated T cells, the B6 anti-BALB/c MLR combination. The effects of CsA on the lymphokine release reaction are shown in Table 4.2. CsA prevents detectable release of both IL2 and IL3 from activated T-cells when induced by antigen or mitogen.

The cells used for this experiment were derived from an MLR between strains that displayed a whole H-2 haplotype difference. It was of interest to determine the relative sensitivity to CsA of class 1 and class 2-MHC-reactive T-cells. Seven day activated T cells which were reactive to defined alloantigens were prepared using MLRs between congenic mouse strains as described in section 4.2.1. Cells prepared in this manner have been previously shown to release IL2 upon antigen or mitogen exposure, and that anti-class 1 MHC and not the anti-class 2 MHC reactive T cells were sensitive to treatment with anti-lyt 2 sera and complement (Andrus et al, 1980). For these experiments cells of each subclass were incubated with 5 µg/ml Con A using the lymphokine release procedure of section 4.2.3B. Table 4.3 shows that CsA inhibits release of IL2 and IL3 from each subclass.

4.3.3 CsA has no effect upon constitutive lymphokine release from tumour cell lines

To determine whether CsA can block the secretion of preformed lymphokine, the effect of the drug on tumour cells which constitutively release lympokine was studied.

The tumour cell lines used were: MLA 144, a gibbon cell line which produces IL2, and WEHI 3B which produces IL3. Tumour cells at 1 x $10^6/cm^2/ml$ cells were incubated with 1 ug/ml CsA for 24 hours at 37°C. At the completion of the culture the supernatants were harvested and tested for lymphokine activity. Control cultures were incubated without the drug. The results, presented in Figure 4.1 show that lymhokine release from either MLA 144 or WEHI 3 was not affected by CsA in that time period.

4.3.4 CsA has little effect on lymphokine release from activated T cells in the 2 hour period following antigen or Con A exposure

In section 4.3.3 it was established that CsA does not affect lymphokine release from tumour cells which produce IL2 or IL3 constitutively. One implication of this is that CsA has no effect upon release of already synthesised lymphokine. To confirm this result, and to ensure that it was not peculiar to tumour cells, an experiment was designed to test the ability of CsA to inhibit lymphokine release from activated T cells for the 2 hour period after an initial triggering stimulus from either Con A or antigen.

T cells were seven day-activated B6 anti-BALB/c prepared as described in section 4.2.1 and 4.2.2. The cells were suspended at 1 x 10^6 cells/ml in EMEM containing 10^{-4}M 2-ME and incubated in 3 ml volumes in culture tubes (Falcon,

2003). Control tubes were incubated for 2 hours with 5 µg/ml Con A, washed and 3 mls of fresh medium added before a further 2 hour incubation at 37°C. A CsA control received 1 µg/ml CsA for both 2 hour culture periods and the test sample was stimulated with Con A in the first 2 hour period and received 1 ug/ml CsA for the second 2 hour culture. The supernatants harvested from the second two hour culture in each group were assayed for IL2 and IL3.

Results of two such experiments are shown in Table 4.4 These results indicate that CsA has little effect upon lymphokine release by activated T-cells for the 2 hour after the reaction has started.

Identical experiments were also performed using P815 as the lymphokine trigger. These results (Table 5) show the same pattern of inhibition as that observed for Con A triggering.

4.3.5 CsA acts before the transcription of IL3-encoding mRNA

Having established that CsA does not inhibit the release of synthesized lymphokine from the cell, it was of interest to determine whether the drug acted before lymphokine encoding mRNA was produced. To examine this question hybridization experiments were performed using the IL3 mRNA probe developed by Fung et al (1984).

In a typical experiment 1 x 10^8 seven day B6 anti-BALB/c activated T cells were used in each of three groups;

1) T cells without stimulation

2) T cells plus 5 µg/ml Con A

3) T cells plus Con A plus 1 µg/ml CsA

Each group of 10^8 cells was incubated in 50 mls of medium in a test tube. The cells were gassed in a 7% O_2, 10% CO_2 and 83% N_2 gas mixture and incubated for 2 hours in a 37°C waterbath. At the end of 2 hours the cells were washed twice and fresh medium without Con A was added to each group. CsA was present in the medium added to the cells in group 3. Following a further 3-4 hour incubation period, the cells were washed twice in PBS and total mRNA prepared by the procedure described in section 3.2.5.

The RNA preparations were electrophoresed on formaldehyde-agarose gels, blotted to nitrocellulose and hybridized with a murine IL3 c-DNA probe to detect IL3 mRNA. The results, which are typical of three separate experiments are shown in Fig 4.2. The only detectable region of complementary RNA was found in lane 2, which contained mRNA from the Con A triggered cells. The position of the band indicated a molecular weight of 1.1 kb, similar to that described by Fung et al, (1984) for the IL3 message. This result indicates that CsA acts prior to synthesis of lymphokine encoding mRNA.

4.3.6 CsA added to activated T cells after exposure to antigen significantly alters the time course of lymphokine release

In the time course of lymphokine production described in section 3.3.4 lymphokine accumulated for approximately 10 hours before tapering off to a plateau. It was possible given the information from the preceding sections of this chapter, to test whether the production of lymphokine over this period required repeated stimulation of the T cells via the CsA-sensitive step or whether one exposure to antigen, stimulated lymphokine secretion for a 10 hour period.

To examine this question lymphokine release was initiated in seven day activated-B6 anti-BALB/c T cells using the procedure described in section 4.2.3.A. After two hours incubation, each culture was washed twice with medium and supplemented with either fresh medium or medium containing 1 μg/ml CsA. The time course of IL3 release was then followed in each group. From Figure 4.3 it can be seen that IL3 release in the cultures which received CsA continues for a further 3-4 hours before reaching a plateau. However, the control cultures continue to produce IL3 for a further 8 hours. This indicates that continued lymphokine production from activated T cells requires repeated stimulation through a CsA sensitive step.

4.3.7 Lymphokine release decreases at a consistent rate irrespective of when CsA is added after an initial triggering period

An experiment was performed which examined the effect of CsA on the rate of IL3 production from T cells which had been previously committed to lymphokine production for 2, 4 or 8 hours.

Four replicate 3 ml cultures containing 1.5 x 10^6 seven day-activated T cells and 6 x 10^6 UV-irradiated P815 were incubated at 37°C for 2, 4 or 8 hours before being washed and divided into two separate cultures. In one group (the control), the supernatant was harvested every 2 hours and the cell pellet was washed and re-fed with fresh medium. This procedure of repetitive supernatant removal and addition of fresh medium was performed on 5 consecutive 2 hour periods. The procedure for the other culture group was identical except that medium containing 1 µg/ml CsA was added to the cells after each wash. In this way the rate of IL3 production per 2 hour period in the presence and absence of CsA was determined.

The result of assaying each supernatant generated in the above experiment for IL3 is shown in Figure 4.4 . In the control cultures IL3 is released at the approximate rate of 3 $\log_{10}$ units/2 hours for the first 8 hours after the

initial wash. From that point the rate of release decreased slowly until, by 18 hours, very little IL3 was being synthesized. When CsA was included in the medium the IL3 titre varied between 50-100% of control values over the first 2 hours. Following this, the titre declined approximately 10-fold for each ensuing 2 hour period and was undetectable 8 hours after drug exposure. This pattern of action was identical whether CsA was added at 2, 4 or 8 hours after antigen stimulation, and therefore suggests that the CsA sensitive-step maintains a constant rate of IL3 production throughout the normal period of lymphokine release.

4.4 DISCUSSION

CsA inhibits the release of IL2 and IL3 from activated T cells responding to Con A or alloantigen (section 4.3.2). In the previous chapter, it was suggested that the lymphokine release reaction could be divided into a number of stages: the binding of antigen; the transmission of an antigen signal; the transcription of lymphokine-encoding mRNA, and the translation and export of active lymphokine. The inhibitory effect of CsA could therefore be expressed at one or more of these steps. Experiments presented in sections 4.3.3. and 4.3.4. demonstrate that constitutive release of lymphokine from tumour cell lines was not affected by CsA. These findings indicate that CsA does not inhibit the export of active lymphokine, has no inhibitory effects upon translation of lymphokine and has no direct effects upon the lymphokine encoding mRNA. In section 4.3.5 it was found that IL3-encoding mRNA was not produced in CsA blocked lymphokine releasing cells. IL2 encoding mRNA is also absent in CsA treated cells (Granelli-Piperno et al, 1984). These experiments suggest that CsA affects lymphokine release at a site before synthesis of lymphokine coding mRNA, blocking either transmission of the antigen signal or the binding of the T cell to its target. However, CsA does not inhibit the expression of cytotoxic activity displayed by either bulk cultures or cytotoxic T cell clones (Andrus and Lafferty,

1982; Bunjes et al, 1981; Orosz et al 1983). Since expression of cytotoxic activity requires the binding of the T cell to its target, it follows that the drug cannot inhibit antigen binding to the T cell.

A possible objection to this argument could be that the cytotoxic cell and the lymphokine-secreting cell represent two distinct cell types: antigen binding by the cytotoxic cell would be unaffected by CsA, whereas the drug would block antigen binding by lymphokine secreting cells. This hypothesis cannot satisfactorily explain the observation that the cytotoxic and lymphokine release activities of an activated T cell population display a common pattern of target cell cross reactivity (Andrus and Lafferty, 1981; Sinickas et al,submitted for publication), suggesting that if they are not the same cell then they at least share a common antigen binding receptor. Moreover, the capability of single cells to possess both cytotoxic and lymphokine release function, has been demonstrated by numerous reports of multifunctional T cell clones (Kelso and Glasebrook, 1984; Andrus et al, 1984).

An important variation of the above argument is the claim of Palacios and Moller (1981) that CsA blocks binding of class 2-MHC restricted T cells to the class 2 MHC antigen. This postulate is based on the idea that only class 2-MHC-restricted T cells produce IL2, a growth factor essential for activation and growth of class 1-MHC-

restricted T cells. However, IL2 is produced by class-1-MHC reactive T cells (Andrus and Lafferty, 1981; Swain, 1983) and lymphokine production by these cells is also inhibited by CsA (Table 4.3). Indeed Palacios and Moller have based their concept on experiments which show that CsA blocks IL2 production in a system which only involves class 2 MHC antigen recognition. These experiments do not contradict the conclusion that CsA blocks the reactivity of both class 1 and class 2 restricted T cells.

If CsA does not inhibit antigen binding and if it does not affect the synthesis or export of lymphokine from the activated T cell, then the drug must interfere with the transmission of the antigenic signal to the nucleus of the cell. Given this conclusion it would be expected that release of all lymphokines controlled by this signal would be inhibited. Lymphokines other than IL2 and IL3 which are not released by activated T cells in the presence of CsA include, γ-interferon (Kalman and Klimpel, 1983), MAF, macrophage migration inhibition factor (MIF) (Thomson et al, 1983), and CSF (Kaufman et al, 1984). In each case CsA has no effect on the assay of the lymphokine activity within the same dose range. These studies suggest that lymphokine activities which are not released in the presence of CsA constitute a set of antigen controlled lymphokines. All lymphokines tested so far belong to this set.

This designation of the site of action of CsA has also allowed a closer analysis of the role of antigen in

lymphokine release. Lymphokine release from activated T cells exposed to antigen will continue for a period of about 10 hours before subsiding. When CsA is added, the rate of lymphokine release remains constant for about 2 hours before rapidly decreasing. This suggests that continued antigen signals are required to maintain the release of lymphokine from the T cell.

Kronke et al, (1984) examined the effects of CsA on the expression of a number of genes induced in the tumour cell line Jurkat by Con A plus PMA. IL2 release was inhibited whereas other non-lymphokine but signal dependent genes such as those for HT-3 and the IL2 receptor were unaffected. This result implies that CsA does not inhibit all the effects of antigen signalling. It also suggests that CsA acts at a site after the antigen signal has diverged into a number of branches.

Inhibition of the antigen signal could also explain why CsA blocks primary T cell activation by alloantigen (Andrus and Lafferty, 1982; DosReis and Shevach, 1982; Hess and Tutschka, 1980), mitogen (Larsson, 1980; DosReis and Shevach, 1982; Weisinger and Borel, 1979; Hess and Tutscha, 1980), and haptens (DosReis and Shevach, 1982). However this theory is mitigated by the observations of Bunjes et al. (1981) and Hess (1985) who have found that addition of exogenous IL2 can restore the proliferative response of T

cells although it does not affect the specific block on induction of cytotoxic T cells. This result could be explained by again assuming a dichotomy in the transmission of the T cell antigen signal. One path, which is CsA-sensitive, controls differentiation in the unprimed cell and lymphokine release in the activated cell. The other CsA resistant signal, may regulate the proliferative response in both the unprimed and the primed cell.

Effects of CsA which cannot be explained by the drugs effect on the T cell antigen signal include inhibition of IL1 release from tumour cells (Andrus and Lafferty,1982), competitive binding to T cell IL1 receptors (Bendtzen and Dinarello, 1984) and inhibitory effects upon antibody responses to some T-independent antigens and B cell mitogenic responses to anti-μ antibody (Kunkl and Klaus, 1980; Dongworth and Klaus, 1982). The latter effects may indicate at least one common signalling mechanism between B and T lymphocytes.

In conclusion, it appears that CsA has effects on T cell activation and lymphokine release and no effects on IL2 mediated proliferation, cell mediated cytotoxicity, or the activities of any known lymphokine. These characteristics are consistent with the observation that CsA acts on the T cell antigen signal. Effects on B cell function cannot be so explained, but could suggest at least one common signalling mechanism between the two lymphocyte subsets.

These conclusions allow us to make a certain prediction concerning the *in vivo* function of this agent. At levels of 100-1000 ng/ml in the tissue fluids, the drug should inhibit T cell priming and so inhibit primary immune responsiveness. The agent will inhibit lymphokine dependent but not cytotoxic functions of activated T cells. The possibility of using these properties to probe T cell function *in vivo* is explored in the next chapter.

TABLE 4.1

CsA does not affect the assay for IL3

Additions to IL3 standard[a]	IL3 activity (log_{10} endpoint titre)
None	3.2[b]
DMSO (3 µl/ml)	3.3
3 µg/ml CsA (in 3 µl/ml DMSO)	3.2
1 µg/ml CsA	3.1
0.1 µg/ml	3.1

[a] Standard IL3 preparation was a 1:10 dilution of a 24 hour WEHI 3 cell supernatant.

[b] Arithmetic mean of duplicate cultures. Expected 95% confidence of titres determined in this manner is ± 0.15 log_{10} units.

TABLE 4.2

Effect of CsA on the release of IL2 and IL3 from activated T cells.

Culture[a]	Lymphokine activity (log_{10} endpoint titre)	
	IL2	IL3
T only	≤0.3	≤0.3
T + Con A + DMSO	1.65	3.7
T + Con A + 1 µg/ml CsA	≤0.3	≤0.3
T + P815 + DMSO	1.65	2.5
T + P815 + 1 µg/ml CsA	≤0.3	≤0.3

[a] 1.5×10^6 B6 anti-BALB/c seven day-activated T cells were stimulated with either Con A or 6×10^6 P815 in 3 ml EMEM + 2-ME as described in section 4.2.3.
The supernatants were harvested after 5 hours incubation at 37°C and assayed for both IL2 and IL3 (section 4.2.4)

TABLE 4.3

Release of lymphokine from both class 1 and class 2-MHC alloreactive T cells is sensitive to CsA

Specificity of T cells [a]	CsA (1 µg/ml)	Lymphokine activity (log_{10} endpoint titre)	
		IL2	IL3
Anti-K^k			
B10.AQR anti-B10.A	-	2.0	3.1
	+	≤0.3	≤0.3
Anti-I^q			
B10.AQR anti-B10.T(6R)	-	2.2	3.2
	+	≤0.3	≤0.3

[a] $1.5x10^6$ seven day-activated T cells which derived initially from the specified MLR's were restimulated with Con A as described in section 4.2.3B. Cell-free supernatants were collected 5 hours after removal of Con A and assayed for IL2 and IL3.

TABLE 4.4

CsA has no effect on lymphokine release from activated T cells pre-exposed to Con A

Culture mixture in first 2 hours[a]	Presence of CsA in 2nd 2 hours	Lymphokine activity in supernatant from 2nd 2 hour period (Log_{10} endpoint titre)	
		IL2	IL3
1)			
T only	-	≤0.3	N.D.[b]
T + Con A	-	1.3	N.D.
T + Con A + CsA	+	≤0.3	N.D.
T + Con A	+	1.3	N.D.
2)			
T only	-	≤0.3	≤0.3
T + Con A	-	1.8	2.4
T + Con A + CsA	+	≤0.3	≤0.3
T + Con A	+	1.6	2.0

[a] Seven day-activated T cells were incubated for 2 hours in EMEM + 2-ME with Con A (5µg/ml) or CsA (1 µg/ml) as indicated. At the end of this culture period the cells were washed twice and then supplemented with fresh medium or medium containing 1 µg/ml CsA where indicated.

[b] Not done.

TABLE 4.5

CsA has no affect on lymphokine release from activated T cells pre-exposed to antigens

Incubation mixture for first 2 hours[a]	Presence of CsA for 2nd 2 hours	Lymphokine activity in supernatant from 2nd 2 hour period Log_{10} endpoint titre	
		IL2	IL3
T + P815	-	1.55	2.7
T + P815 + CsA	+	≤0.3	≤0.3
T + P815	+	1.3	2.4

[a] $1.5x10^6$ seven day-activated B6 anti-BALB/c T cells were incubated with 6×10^6 UV-irradiated P815 in 3 mls EMEM using the method described in section 4.2.3A. After 2 hours these cells were washed twice in EMEM before addition of 3 mls of fresh medium with or without 1 µg/ml CsA. The supernatant was harvested after a further 2 hour culture and assayed for IL2 and IL3 activity.

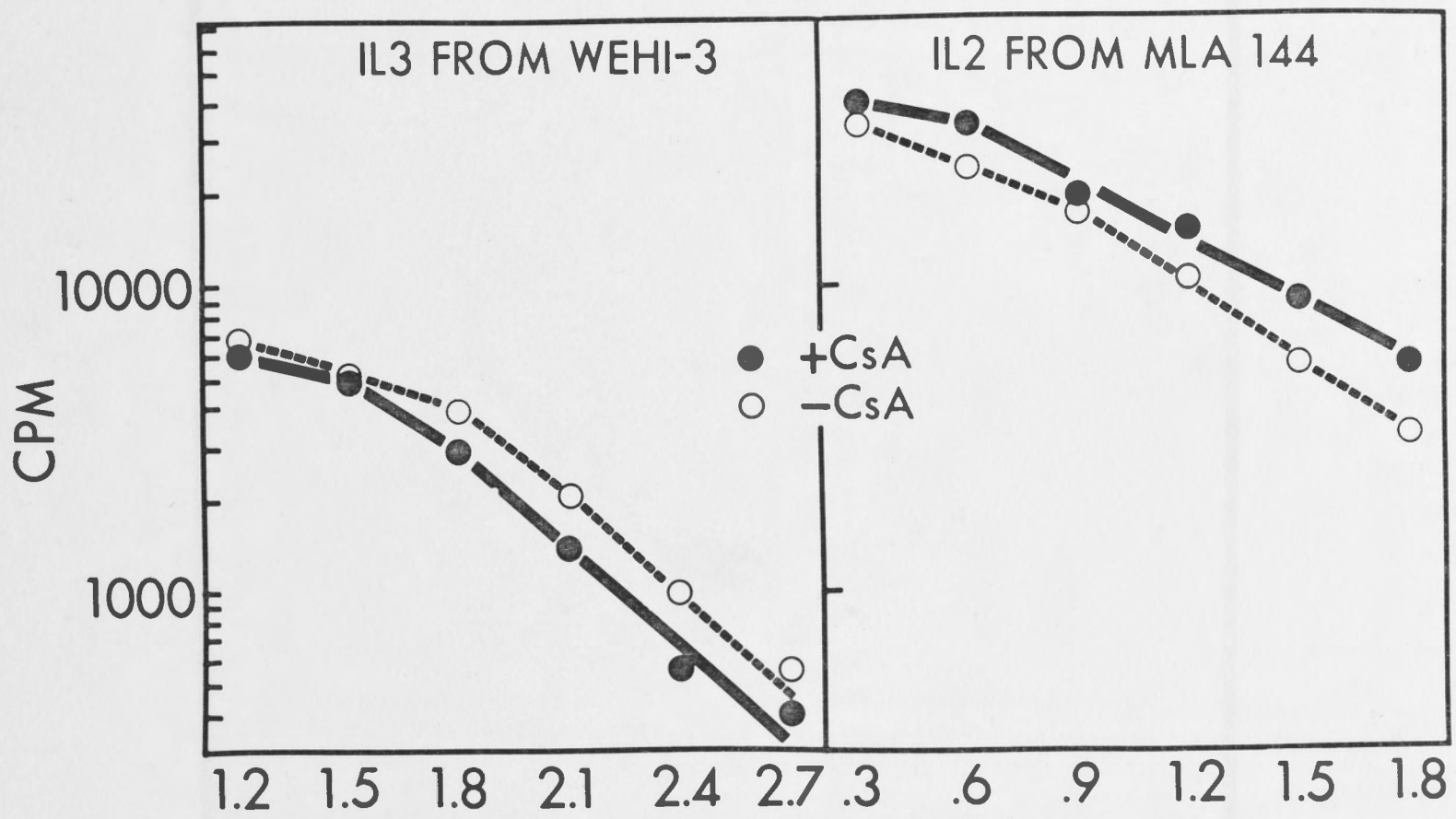


Figure 4.1. The effect of CsA on constitutive lymphokine release from tumour cell lines. The cell lines WEHI 3 and MLA 144 were incubated at 10^6 cells/ml for 24 hours with and without 1 µg/ml CsA. The WEHI 3 supernatants were assayed for IL3 on the 32D cell line and the MLA 144 derived supernatants were tested for IL2. Each point is the mean c.p.m. from duplicate cultures.

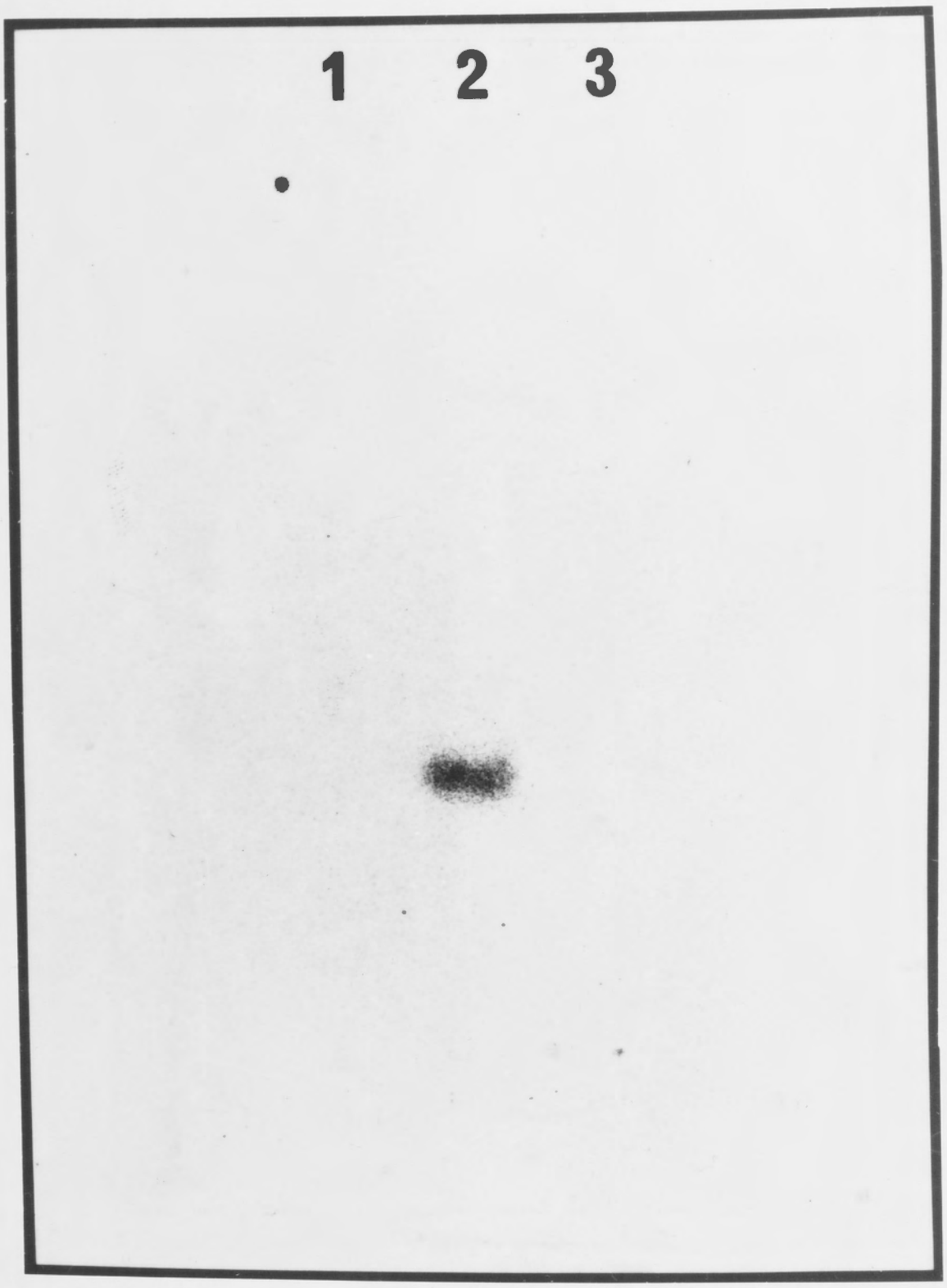


Figure 4.2. The effect of CsA on IL3 mRNA levels. 10^8 seven day-activated T cells were incubated for 2 hours in 50 ml EMEM containing 10^{-4}M 2-ME at 37°C either; alone (lane 1), with 5 µg/ml Con A (lane 2), or with 5 µg/ml Con A and 1 µg/ml CsA (lane 3). These cells were then washed and reincubated a further 4 hours. The cells cultured with CsA also received 1 µg/ml CsA in this second culture period. At the end of culture total mRNA from each group was extracted, and hybridized with a murine cDNA probe as described in section 4.2.6.

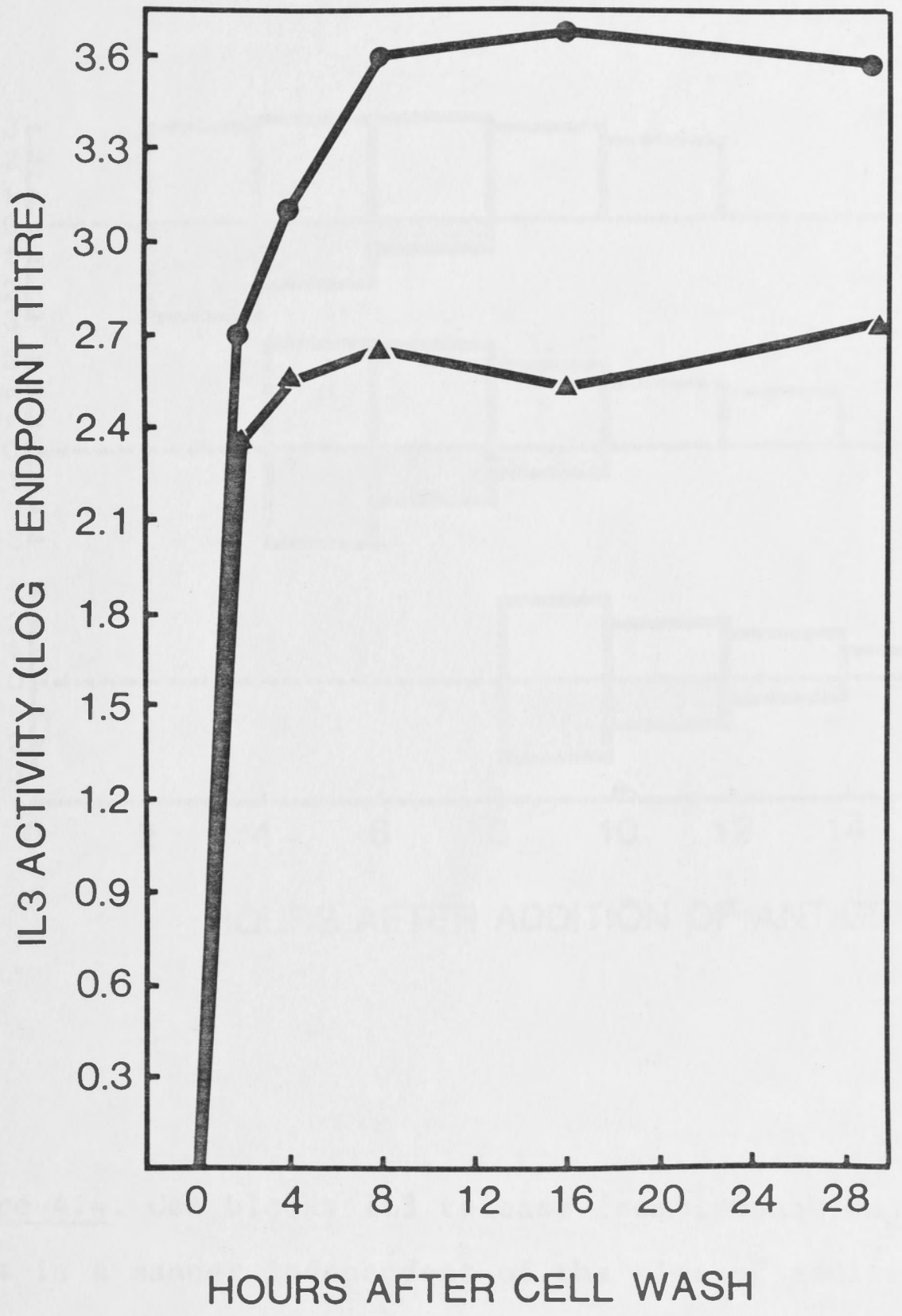


Figure 4.3. Time course of IL3 release after addition of CsA to antigen-triggered activated T cells. Seven day-activated T cells were incubated with UV-irradiated P815 for 2 hours as described in section 4.2.3A. Following this incubation, cells were resupplied with fresh medium (●) or medium containing 1 µg/ml CsA (▲). Samples were harvested at various times and assayed for IL3 activity.

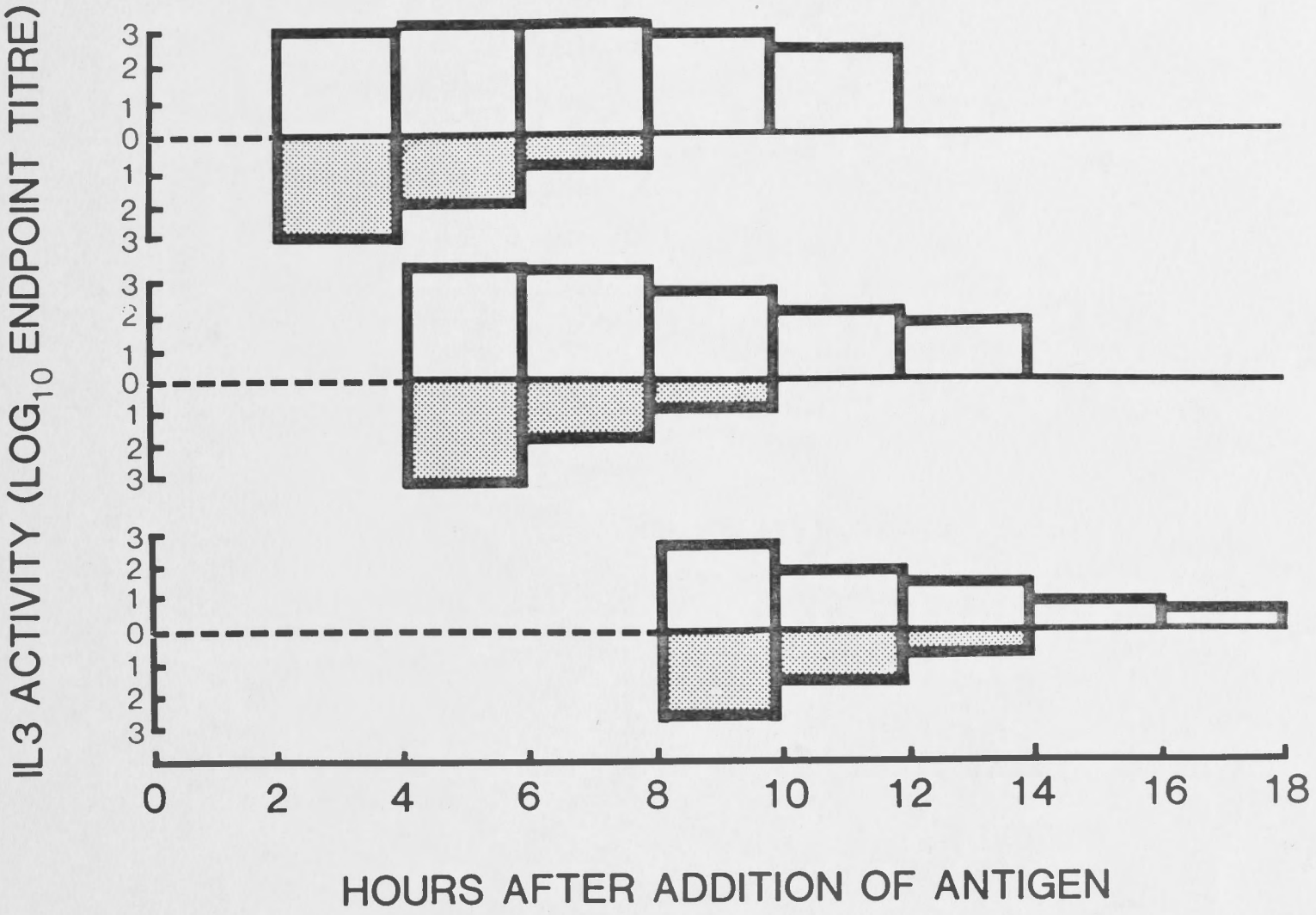


Figure 4.4. CsA blocks IL3 release from lymphokine releasing cells in a manner independent of the time of addition. Seven day-activated T cells were incubated with UV-irradiated P815 as described in section 4.2.3A. In the top panel cells were incubated for 2 hours, in the middle panel 4 hours, and in the lower panel 8 hours, before being washed and supplemented with fresh medium (open columns) or medium containing 1 µg/ml CsA (hatched columns). The supernatant was removed every 2 hours for IL3 assay and fresh medium added to the same cells to continue culture. The procedure of repeated harvest every 2 hours was completed on 5 occasions. The IL3 titres obtained from each 2 hour supernatant are represented as columns in the above figure.

CHAPTER 5

T CELL FUNCTION IN VIVO

5.1 INTRODUCTION

A number of immune functions are mediated by T cells reactive to class 1 MHC antigens. Such functions include graft versus host reactions (GVHR)(Sprent and Korngold, 1981) graft rejection (Prowse *et al*, 1983) and DTH reactions (Ada *et al*, 1981). Cells of this specificity have been shown *in vitro* both to be cytotoxic for antigen bearing target cells and to release a number of lymphokines in response to antigenic or mitogenic stimulation (Andrus *et al*, 1984; Swain, 1981). The relative contributions of these two activities to the *in vivo* function of class 1-MHC reactive T cells is unclear. CsA, which inhibits lymphokine release from activated class 1-MHC reactive T cells but has no effect on their cytotoxic activity (Andrus and Lafferty, 1982. Bunjes *et al*, 1982), offers the potential for use *in vivo* to discriminate between these two functions.

In this chapter this potential is examined using two models; a local inflammatory reaction induced by transfer of activated alloreactive T cells to mouse hind footpads, and the rejection of cultured islet allografts by transfer of sensitized cells to the graft recipient. In both systems biological activities of the class 1 MHC-reactive T cells were inhibited by CsA and therefore appear to be lymphokine dependent.

5.2 MATERIALS AND METHODS

5.2.1 Tumour cell lines

The tumour lines R1(TL+)($H\text{-}2^k$), P815 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) were maintained by weekly passage as described in section 2.4.

5.2.2 Preparation of activated T lymphocytes

Seven day-activated T cells reactive to the K^k alloantigen were prepared from an MLR between the congenic strains B10.AQR (K^k) and B10.A (K^q) using the methods described in sections 2.8 and 2.9. T cells reactive to $H\text{-}2^d$ alloantigens were similarly prepared except that 3.16×10^5 γ-irradiated (2000 rads) P815 were used as stimulator cells in the MLR.

5.2.3 Assay for cell mediated cytotoxicity

Measurement of the cytotoxic activity of cell preparations using ^{51}Cr release from target cells was performed as described in section 2.13. All results are expressed as $\log_{10}$ cytotoxic units.

5.2.4 Lymphokine release

Con A was used to induce lymphokine release from seven-day activated B10.AQR anti-B10.A T cells using the method

described in section 4.2.3B. Supernatants were assayed for IL2 activity on Con A blasts as described in section 2.10.

5.2.5 Antiserum treatment of cell populations

Treatment of cells with monoclonal anti-Thy 1 serum (Olac, England) and anti-lyt 2 serum, (ascites fluid from HO-2.2 hybridoma, Gottlieb et al, 1980) was as follows.

Cells at 10^6/ml were incubated for 1 hour at 4°C in EMEM containing the optimum antisera concentration. The cells were washed once in fresh EMEM and resuspended in a 1/20 dilution of rabbit complement (C')(Cedarlane, Ontario) in EMEM. This suspension was incubated for 1 hour at 37°C before being washed twice and resuspended in fresh EMEM plus 10^{-4}M 2-ME.

5.2.6 CsA

When used in vitro the drug was dissolved at 1 mg/ml in DMSO and diluted in medium to a final concentration of 1 µg/ml. Control cells were treated in an equivalent volume of DMSO in medium. For animal treatment the drug was dissolved at 25 mg/ml in olive oil and injected subcutaneously at a dose rate of 75 mg/kg/day.

5.2.7 Assay for local GVHR

Five million seven-day activated T cells in 50 µl EMEM

were injected into the left hind footpad of B10.A mice. The right footpad received a control injection of medium only. Each hind foot pad was measured at a number of time intervals using a dial gauge caliper (Mitutoyo, Japan). Results are expressed as percent increase over control foot and calculated using the following formula:

$$\frac{\text{thickness (left - right foot)}}{\text{thickness right foot}} \times 100$$

5.2.8 Immunization of mice

Spleen cell suspensions were prepared from CBA mice 10 days after the intraperitoneal injection of 2.5×10^7 P815. The cytotoxic activity of the spleen cells was measured on P815 target cells. The cytotoxic activity of the spleen cells used in this study varied from 5.4 to 6.1 $\log_{10}$ C.U./spleen.

5.2.9 Preparation, culture and transplantation of islet tissue

The method for preparation of pancreatic islets from BALB/c mice pancreas is described in section 2.15.

CBA mice made diabetic by the intravenous injection of streptozotocin (250/mg/kg; Calbiochem-Behring), received

transplants of 350-400 islets under the kidney capsule. Animals usually became normoglycaemic in less than 7 days. Mice in which normoglycaemia was restored and maintained for at least 30 days post transplant were used in this study. Spontaneous graft rejection was never seen in transplanted animals.

5.2.10 Transplantation of islet clusters and activated T cells

A cultured BALB/c islet cluster was mixed with 4-10 x 10^5 activated T cells in normal CBA mouse plasma. After clotting by the addition of powdered thrombin the clot was transplanted under the kidney capsule of CBA mice. In some experiments the clot was formed by adding 5 µl of normal CBA blood to the mixture of islets and T cells and allowing this to clot. Animals were killed 3-4 days after transplantation.

5.2.11 Preparation of CBA T cells devoid of reactivity to BALB/c alloantigens

CBA lymph node cells were injected intravenously into gamma-irradiated (850 rads) BALB/c mice. The thoracic ducts of the mice were cannulated and 20 hours later the thoracic duct lymphocytes collected over the following 24 hour period. The cells collected were stimulated with B6 spleen cells in an MLR according to the method described in section 5.2.2.

5.3 RESULTS

5.3.1 Selective in vitro effect of CsA on the function of class 1 MHC-reactive T cells

Seven day activated B10.AQR anti-B10A (anti-K^k) T cells are both cytotoxic and capable of releasing IL2 when appropriately triggered. In each case function is dependent upon a Thy 1^+, lyt 2^+ cell (Table 5.1).

The same cell population produces no detectable IL2 when exposed to Con A in the presence of CsA. In contrast the cytotoxic activity measured on R1(TL+) target cells was unchanged in the presence of CsA (Table 5.2).

5.3.2 CsA inhibits the local GVHR

Seven day-activated T cells induce significant footpad swelling 24 hours after transfer to mice bearing the target antigen (B10.A)(Figure 5.1 and 5.2A). As with IL2 release and cytotoxic activity, this function is also dependent upon a Thy 1^+, lyt 2^+ cell (Figure 5.1). The reaction is antigen specific since cells injected into the footpad of syngeneic (B10.AQR) mice do not induce a footpad response (Figure 5.2A). The treatment of the cells with CsA for 1 hour prior to injection into the footpad of normal B10.A animals delayed the onset of the GVHR, a response becoming evident by 10-20 hours after the injection of the cells

(Figure 5.3B). Despite the delay the intensity of the reaction was the same. Cells *in vitro* regain their IL2 release capacity within 3 hours of removal of the drug (Table 5.3). The injection of CsA treated cells into the footpad of B10.A mice which had been treated with CsA on days -1,0 and 1 caused no reaction (Figure 5.3.B).

The complete inhibition of the footpad GVHR in CsA treated animals suggests that this immune response is dependent upon lymphokine release from class 1 MHC-reactive T cells.

5.3.3 *CsA inhibits the rejection of cultured islet allografts*

Cultured BALB/c islet allografts, which are accepted when transplanted to normal CBA recipient mice, can be rejected by the passive transfer to the recipient of specifically sensitized spleen cells (Prowse *et al*, 1983). Rejection of the cultured grafts is dependent upon the presence of lyt 2 bearing cells in the transferred population (Prowse *et al*, 1983; Warren *et al*, in press).

Diabetic CBA mice become normoglycaemic in approximately 7 days following transplantation with cultured BALB/c islets (Figure 5.3A). After a period of normoglycaemia (>30 days) half the total group of mice (6) were treated with CsA. One day later these mice and 6 grafted but untreated mice

received 5 x 10^7 CBA anti-P815 spleen cells. CsA treatment was continued daily for one week after the injection of the cells. The CsA treated mice remained normoglycaemic during this period (Figure 5.3A), whereas all the control mice became hyperglycaemic (Figure 5.3A). Reversion to hyperglycaemia in this transplant system indicates graft rejection. These results therefore, demonstrate that CsA inhibits graft rejection triggered by the transfer of sensitized cells.

CsA treatment was terminated in 4 mice, upon which 2/4 rejected their grafts. A third went through a rejection crisis then recovered and the forth remained normoglycaemic (Figure 5.3B). The two animals which did not reject their grafts were challenged with 5 x 10^7 BALB/c spleen cells 25 days after cessation of CsA treatment. In both cases graft rejection ensued (data not shown), indicating that a state of donor-specific graft tolerance had not developed in these animals.

This finding indicates that the sensitized cells persist in the recipient mice and in some cases are able to function by rejecting the grafts upon the removal of CsA.

5.3.4 CsA inhibits graft rejection by sensitized cells placed adjacent to the graft

The experiments outlined above show that the rejection

of cultured islet allografts is lymphokine dependent. It is unclear whether the lymphokine is required for the recirculation of the cells or for mounting an inflammatory response at the graft site. To distinguish between these possibilities graft rejection was induced in a model where T cell recirculation was not required. CBA lymph node cells were activated in vitro against P815 and homogeneous populations of T cells generated.

These cells (with or without CsA treatment) were mixed in CBA plasma with a single 7 day cultured BALB/c islet cluster. The plasma was clotted with thrombin and transplanted under the kidney capsule of CBA mice (with or without CsA treatment). The grafts were examined 3-4 days after transplantation. With one exception, all the grafts in the CsA treated animals contained islet tissue when examined by histology. In contrast none of the grafts in the control mice contained islet tissue (Table 5.4). The graft which rejected in a CsA treated animal was in an experiment using very high levels of cytotoxic activity (6.5 C.U./cluster). CBA lymph node cells activated against a third party alloantigen (B6, H-2^b) rejected 3/4 cultured BALB/c islet allografts. However, where these cells have been selectively depleted of reactivity to alloantigens shared between B6 and BALB/c, they are unable to destroy the cultured BALB/c grafts (Table 5.4). This finding indicates that the graft is not destroyed by the non-specific interaction of the islet tissue, activated T cells and the clot.

Thus under conditions where lymphocyte recirculation is not required, CsA still inhibits the rejection process demonstrating the lymphokine dependence of this immune function.

5.4 DISCUSSION

The selective inhibitory effect of CsA upon the function of class 1-MHC reactive T cells is apparent from Table 5.2. The drug strongly inhibits IL2 release while having no affect upon the cells' cytotoxic activity. Release of IL2 is used here as a general indicator of the effects of CsA on lymphokine release. As discussed in previous chapters activated T cells release many lymphokine activities including factors with an obvious role in inflammation, such as phagocyte chemotaxis and macrophage activation. The release of these factors, like IL2, is inhibited by CsA (Kalman and Klimpel, 1983; Thomson *et al*, 1983; Kaufman *et al*, 1984).

Class 1-MHC reactive T cells will induce footpad swelling (local GVHR) within 24 hours of injection into the footpads of mice bearing the priming antigen (Figure 5.2A). The reaction was dependent upon a cell of $Thy1^+$, $lyt2^+$ phenotype (Figure 5.1). The reaction however is sensitive to CsA, as treatment of the cells with the drug prior to injection results in a delay in the onset of the reaction. This suggests that the local GVHR is lymphokine dependent and also demonstrates that cells will function *in vivo* after the reversal of the effects of CsA. This is also true *in vitro*, as cells treated with CsA and then washed and resuspended in medium free of CsA regain their capacity for lymphokine production within 3 hours (Table 5.3).

Pretreatment of both cells and recipient animals with CsA completely inhibits the development of footpad swelling. From these results it appears that induction of the local GVHR is dependent upon lymphokine release and occurs independently of the cells' cytotoxic activity.

The acute rejection of cultured islets is dependent upon a lyt 2^+, and therefore presumably class 1-MHC-reactive (Swain, 1982), T cell population (Prowse et al, 1983; Warren et al, in press). This rejection process is inhibited by CsA (Figure 5.3) again demonstrating a dependence upon lymphokine release. It could be argued that when effector cells are introduced into the systemic circulation of CsA treated mice, they cannot recirculate and reach the graft site. However, under conditions where lymphocyte recirculation is not required, CsA still inhibits the rejection process (Table 5.4). The rejection of these grafts is the result of antigen specific recognition since activated T cells which have been depleted of reactivity to graft antigens, are unable to reject grafts. Since lymphokine release is CsA sensitive and cytotoxic activity is not, these data suggest that graft rejection mediated by class 1 MHC-reactive T cells is also a lymphokine dependent process.

These findings raise an important question concerning the process of allograft reactivity: Is cytotoxic activity of the effector T cell population irrelevant to the process

of tissue damage? This is a possibility, although the observation that one graft was damaged when the CsA treated T cells had a high cytotoxic activity (Table 5.4) indicates a role for direct cell mediated killing in the rejection process. It is also possible that lymphokine production is required for the expression of cytotoxic activity *in vivo*. IL2 may be required to maintain the cytotoxic cells in an active proliferating state for a sufficient time to cause tissue damage. The proliferation of T cells at the graft site is a feature of rejecting allografts (Ascher *et al*, 1984). Alternatively, γ-interferon production may be required to raise the antigen density on target cells to the level required for efficient killing (Wong *et al*, 1984; Bukowski and Welsh, 1985).

Another possibility is that the cytotoxic activity of the cell population has little to do with the rejection process. According to this hypothesis tissue damage would be indirectly controlled by the T cell. That is, lymphokine production (for example IL3, γ-interferon) by the class 1 MHC-reactive T cell activates inflammatory cells which in turn are the proximate mediators of tissue damage. It should be possible to analyze this situation further by the use of cloned T cell populations that express cytotoxic activity but have lost the capacity to produce one or more of the lymphokines.

In conclusion, the studies reported here emphasize the

potential for using CsA to discriminate between the dual biological activities of class 1 MHC-reactive T cells. The data indicates that induction of a local GVHR, and graft rejection are both dependent upon the lymphokine release function of these cells. The exact nature of this lymphokine dependence is unknown.

Other *in vivo* functions of class 1 MHC-reactive T cells in which the relative contributions of cytotoxicity and lymphokine release are unknown include, skin graft rejection (Loveland and McKenzie, 1983; LeFrancois and Bevan, 1984), tumour graft rejection (Engers *et al*, 1984; North and Bursuker, 1984; Eberlin *et al*, 1982) and virus elimimination (Ada *et al*, 1981). It is anticipated that CsA will be a useful tool in assessing this contribution.

TABLE 5.1

A Thy 1^+, lyt 2^+ cell mediates both IL2 release and cytotoxicity

Treatment of cell population	IL2 activity[a] (log_{10} endpoint titre)	Cytotoxic activity[b] (log_{10}CU/10^6 cells)
C' only	2.9	6.0
Anti-Thy1 + C'	0.6	≤4.9
Anti-lyt2 + C'	0.9	≤4.9

[a] Seven day-activated B10.AQR anti-B10.A (anti-K^k) T cells were triggered to release IL2 following the procedure outlined in section 5.2.4.

[b] Cytotoxicity was measured on R1(TL+) ($H\text{-}2^k$) target cells as described in section 5.2.3.

TABLE 5.2

CsA inhibits IL2 release from class 1 MHC-reactive T cells

Treatment of cell population[a]	IL2 activity[b] (log_{10} endpoint titre)	Cytotoxic activity[c] (log_{10}CU/10^6 cells)
Untreated	2.0	6.15
CsA treated[d]	<0.3	6.14

[a] Seven day-activated B10.AQR anti B10.A T cells prepared as described in section 5.2.2.

[b] 5 µg/ml Con A was used to trigger IL2 release from activated T cells following the method described in section 5.2.4.

[c] Cytotoxic activity of activated T cells was measured on R1(TL+) target cells as described in section 5.2.3.

[d] 1 µg/ml CsA included in IL2 release assay or cytotoxicity assay.

TABLE 5.3

T cells recover lymphokine release capacity following CsA exposure

Cell treatment[a]	IL2 activity (Log_{10} endpoint titre)
0 recovery time[b]	
No pretreatment	1.5
CsA pretreatment, CsA in culture	≤0.3
CsA pretreatment	≤0.3
3 hour recovery time	
No pretreatment	1.85
CsA pretreatment	1.70
6 hour recovery time	
No pretreatment	1.65
CsA pretreatment	1.65

[a] Cells used were seven day-activated B10.AQR anti-B10.A T cells.

[b] Cells were pretreated with 1 µg/ml CsA for 1 hour at 37°C. The cells were then washed twice in EMEM containing 10^{-4}M 2-ME and then resuspended in fresh medium containing 3% CS. These cultures were incubated at 37°C for the indicated recovery time before being washed and stimulated with 5 µg/ml Con A as described in section 5.2.4.

TABLE 5.4

CsA Prevents Graft rejection mediated by Class 1-MHC-reactive T cells

Specificity of cells	Treatment	Cytotoxic activity (C.U./cluster) P815	EL4	Grafts with islet tissue/no. grafts
CBA anti-P815[a]	None	5.5	ND	0/2
CBA anti-P815	None	6.5	ND	0/2
CBA anti-P815	CsA	5.5	ND	3/3
CBA anti-P815	CsA	6.5	ND	2/3
CBA anti-B6	None	≤4.7	5.3	1/4
CBA^{+}anti-B6[b]	None	≤4.7	6.4	4/4

[a] Seven day-activated T cells prepared as described in section 5.2.2.

[b] CBA^{+} indicates that the cells stimulated by B6 spleen cells had been filtered through a BALB/c mouse to selectively deplete reactivity to BALB/c alloantigens (Section 5.2.11).

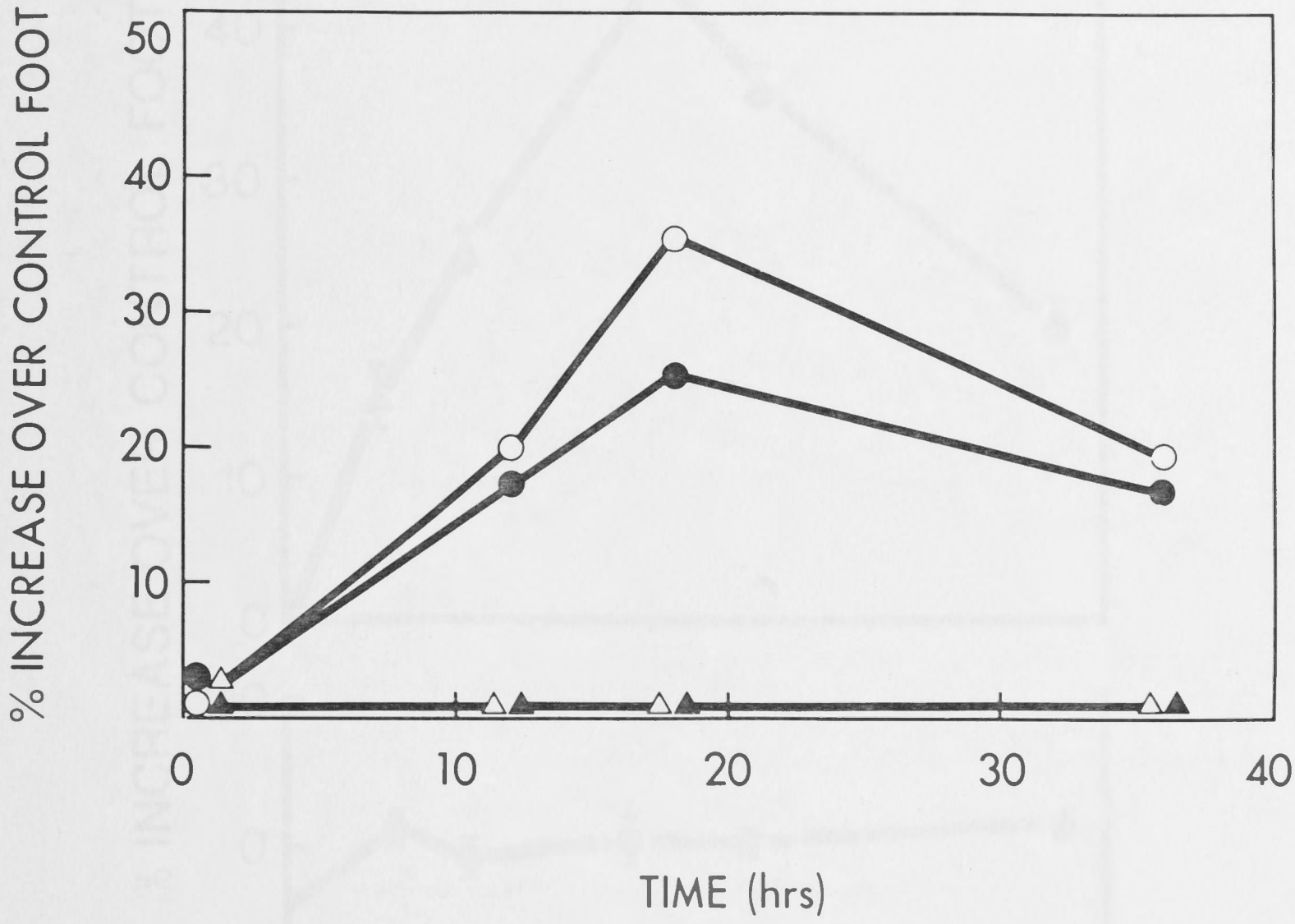


Figure 5.1. The phenotype of alloreactive T cells responsible for footpad swelling. B10.A mice received an injection of 5 x 10^7 seven day-activated B10.AQR anti-B10.A T cells into the left hind footpad. Inoculated cells had been either, left untreated (○), incubated with C' only (●), incubated with anti-Thy 1 and C' (△) or treated with anti-lyt 2 and C'(▲). Each point is the mean increase of 2 animals.

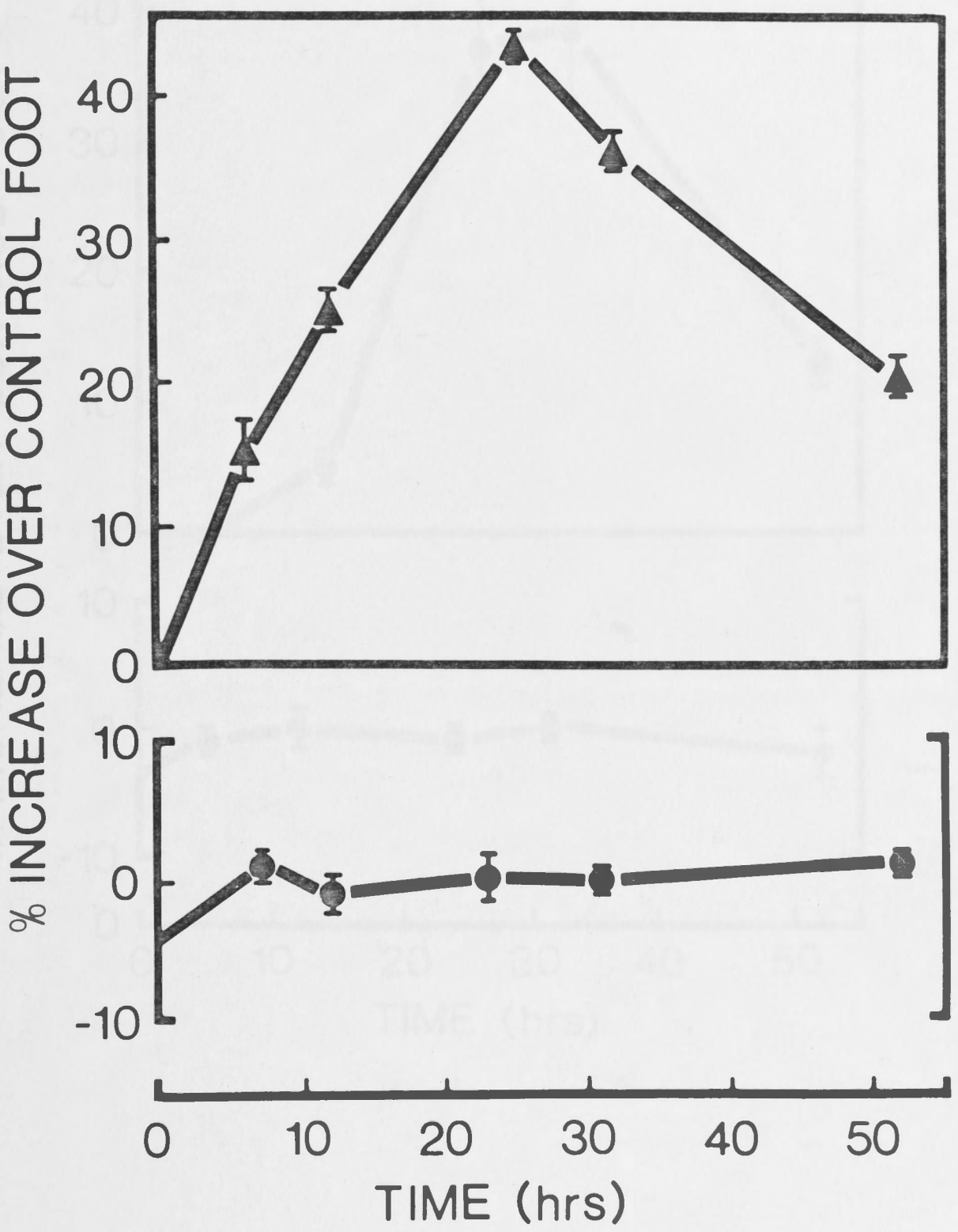


Figure 5.2A. The mean % increase in footpad swelling in 3 B10.A mice (top panel) or 3 B10.AQR mice (lower panel) following the injection of 5 x 10^7 seven day-activated B10.AQR anti-B10.A T cells into the left hind footpad. The bars represent twice the S.E.

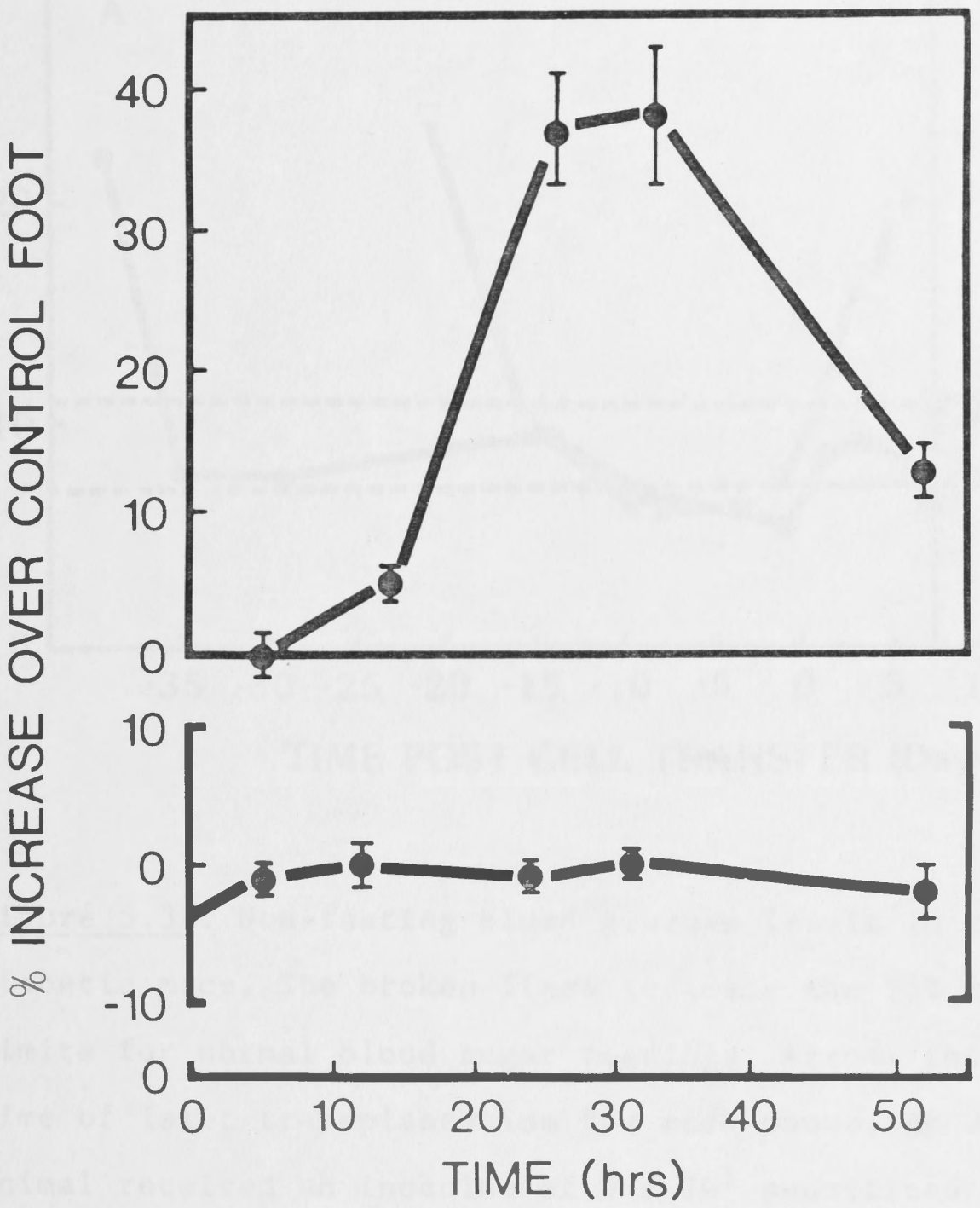


Figure 5.2B. The mean % increase in footpad swelling in 3 B10.A mice following the injection of 5 x 10^7 seven day-activated B10.AQR anti-B10.A T cells pretreated for 1 hour with 1 µg/ml CsA, into the left hind footpad. The lower panel shows the mean % increase in footpad swelling when the same cells were injected into CsA treated recipients. The vertical bars represent twice the S.E. This experiment was performed using the same cell preparation used for Figure 5.2A. Thus, the curves can be directly compared.

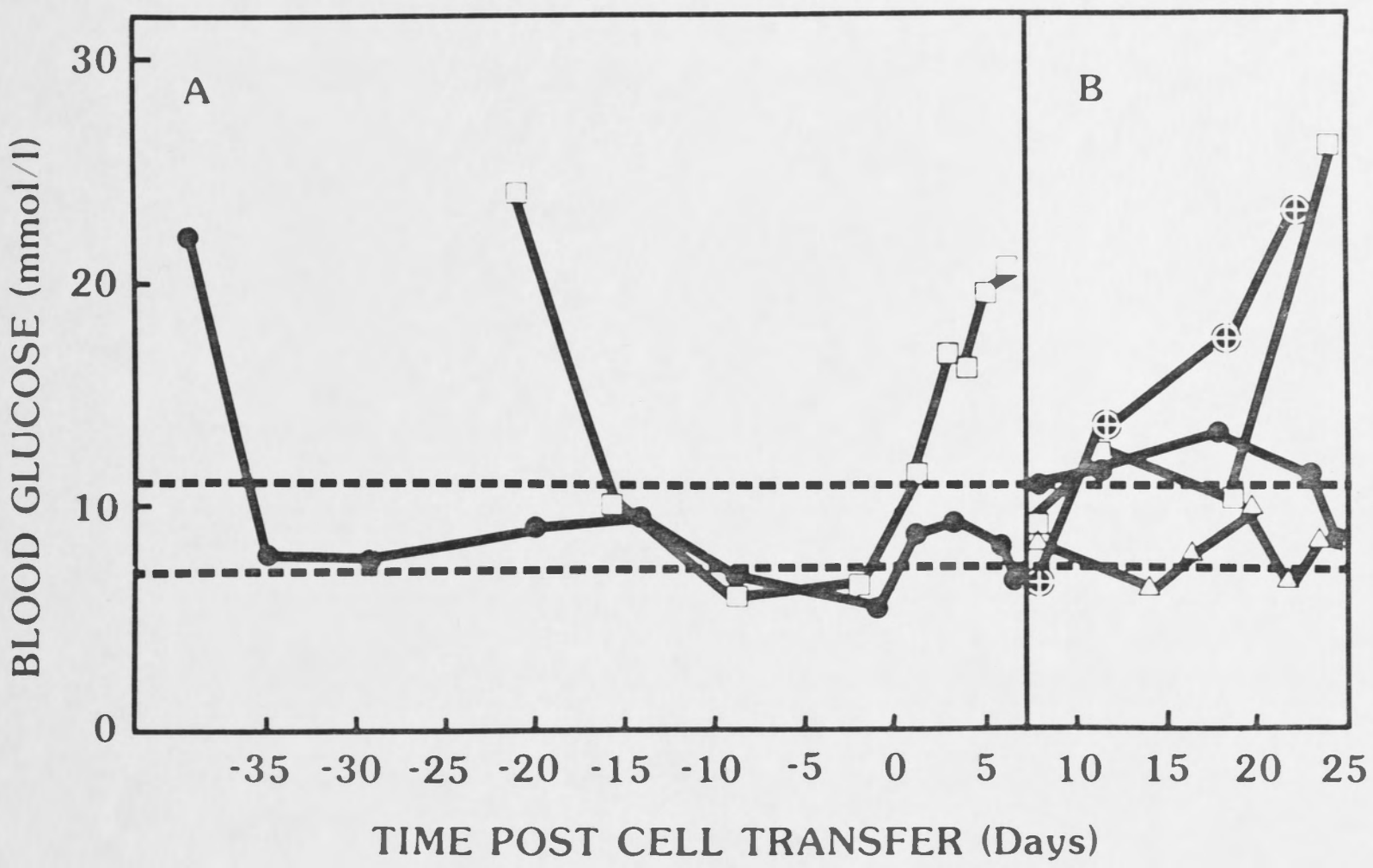


Figure 5.3A. Non-fasting blood glucose levels in two diabetic mice. The broken lines indicate the 95% confidence limits for normal blood sugar readings. Arrows indicate the time of islet transplantation for each mouse. At day 0 each animal received an inoculum of 5×10^7 sensitized CBA anti-P815 spleen cells. The animal indicated by the symbol (□), is representative of six control animals all of which returned to the diabetic state within three days post cell transfer. The animal shown by the sumbol (●), is representative of six animals which received 75 mg/kg CsA on days -1 to 6 post cell transfer. All six animals remained normoglycaemic through this period.

Figure 5.3B. Of the six animals, two were killed for histological examination and the remaining four were monitored for a further twenty days. The blood glucose readings of each animal is shown in this panel. Of the four animals two rejected their graft following withdrawal of CsA treatment.

CHAPTER 6

T CELL DOSE RESPONSE CURVES:

THE TRIGGERING ACTION OF T CELL MITOGENS

6.1 INTRODUCTION

T cell mitogens such as Con A or $NaIO_4$ are often used to activate large numbers of T cells, either to explore the potential responsiveness of a lymphocyte population or to prepare lymphokine-rich supernatants. The attitude of the immunologist to these agents can be summarized into two camps. In the first, lectins and oxidizing agents are seen as pharmacological agents which act directly on the T cell to initiate events leading to activation (Greaves, 1977) The alternative view is that these mitogenic responses mimic the requirements of antigenic stimulation by rendering the cells themselves recognizably antigenic (Kimura and Ersson, 1981). Adopting this latter view leads to the hypothesis that analysis of the mechanisms by which these agents trigger large numbers of lymphocytes can supply important information regarding T cell activation and its antigenic and cellular requirements.

Recent evidence suggests that the second view may be correct. A number of similarities between alloantigenic and mitogenic responses have been described. Alloantigen, lectins and oxidizing agents will only stimulate a response if a specialized accessory cell is present in the cultures. This cell has the adherent properties of macrophages (Greineder and Rosenthal, 1975; Mishell and Miller, 1975; Schilling et al, 1976; Rosenstreich et al, 1976; Novogrodsky et al, 1977). For each stimulating procedure

the accessory cell requirement can be circumvented if a soluble factor, IL1, is supplied exogenously (Larsson and Coutinho, 1980; Lafferty et al, 1980). These observations have given rise to a two signal model whereby T cell activation requires both an antigenic/mitogenic signal and a signal from a soluble co-factor normally delivered by the accessory cell (Lafferty et al, 1980; Larsson and Coutinho 1980). The identical co-factor requirements for each method of stimulation can be explained if it is assumed that syngeneic accessory cells are recognizable to T cells following lectin binding or mild oxidization in a manner which mimics a MHC-disparate cell.

There is, however, indirect evidence that mitogens do act directly on the cell. T cell mitogens can trigger lymphokine release from populations of activated T cells which do not contain accessory cells (Andrus and Lafferty, 1981; Ely et al, 1981). This result suggests that lectin simply binds to the T cell and initiates an antigen equivalent signal. Experiments by Chilson et al (1984) have also shown that mitogenic lectins can bind to some components of the T cell antigen receptor. This interpretation appears at odds with the hypothesis given above which suggests that triggering must always involve presentation of the mitogen on another cell. It is possible however, that mitogen stimulation does involve cellular presentation, and in the case of pure T cell populations these cells cooperate, with one cell presenting

the mitogen to another T cell. In chapter 3 it was found that triggering lymphokine release from activated T cells is dependent solely upon an antigenic signal. Clearly if mitogens act directly on the T cell then the lymphokine titre which follows a period of mitogen stimulation will be directly related to the T cell number. That is, if lymphokine release occurs via the following scheme;

$$T + \text{mitogen} \rightarrow IL3$$

then the relationship between T cell dose and IL3 titre will be of the following form;

$$(IL3) = kN_T \qquad (6.1)$$

where k represents the mean IL3 titre attributable to one lymphokine releasing cell, N_T is the T cell number and (IL3) is the IL3 titre. If the T cells co-operate in the following way;

$$T + T + \text{mitogen} \rightarrow TT_m \rightarrow IL3 \qquad (6.2)$$

where TT_m is a mitogen induced T cell conjugate, then a higher order relation than that shown by equation 6.1 will be found.

In this chapter the cellular requirements for mitogenic triggering of lymphokine release is explored by examining T cell dose response curves.

6.2 MATERIALS AND METHODS

6.2.1 Lymphokine release assays

B6 anti-BALB/c seven day-activated T cells were stimulated with mitogens in 200 µl EMEM containing 10^{-4}M 2-ME. Each culture was prepared in wells of a 96 well microtitre tray (NUNC) and incubated for 6 hours at 37°C in an atmosphere of 10% CO_2; 7% O_2; 83% N_2. All assays were done in quadruplicate and the supernatant IL3 titre measured using the hexosaminidase method to measure the extent of proliferation of the FD IL3-dependent cell line (section 2.12B).

6.2.2 Periodate treatment of cells

T cells or UV-irradiated (section 2.7) EL4 tumour cells were incubated at 10^7 cells/ml with 10^{-4}M $NaIO_4$ in PBS for 10 min at room temperature. Cells were washed twice in EMEM before being resuspended at the required cell concentration.

6.2.3 Pretreatment of cells or plastic wells with Con A

UV-irradiated (section 2.7) EL4 and K562 tumour cells were suspended at 10^7 cells/ml in EMEM containing 10^{-4}M 2-ME and 15 µg/ml Con A and incubated for 15 min at 37°C. The cells were then washed twice in fresh medium and added to the lymphokine release procedure.

NUNC plastic 96-well microtitre trays were preincubated with 100 µl of Con A solution in PBS for 30 min at 37°C. The solutions were then flicked off by rapid inversion of the plate. Each well was then washed twice with 200 µl PBS.

6.2.4 Estimation of cell binding to plastic wells

Each well received 10^4 activated T cells in 100 µl EMEM supplemented with 10^{-4}M 2-ME. These plates were then incubated for 1 hour at 37°C. The liquid contents were then ejected by rapidly inverting the plate. Each plate then underwent three cycles of immersion in PBS followed by flicking off the contents. To estimate the level of cell binding the number of remaining cells was assayed by adding the hexosaminidase reagent p-nitrophenol-N-acetyl-β-D-glucosamine. The method for using this reagent to measure cell numbers is described in section 2.12B. The O.D. obtained for 10^4 T cells was taken as the 100% figure for a percentage binding estimation.

6.3 RESULTS

6.3.1 Mitogenic tiggering of lymphokine release from activated T cells requires T-T interaction

A linear relationship between T cell dose and lymphokine titre is expected for mitogenic triggering if these agents act directly upon the T cell to trigger lymphokine release. If, however, T-T collaboration is required, extra constraints will be placed upon the T cell number and the reaction order will be higher than one.

Figures 6.1 and 6.2 present T cell dose response curves generated following stimulation with a range of mitogens. For the mitogens Con A, leucoagglutinin and $NaIO_4$ the plot of $\log_{10}$ (IL3) versus $\log_{10} N_T$ produces a curve with the slope of the linear section close to 2. This suggests a relation of the following form;

$$\log_{10} (IL3) = 2 \log_{10} N_T + \log_{10} k \qquad (6.2)$$ or,

$$(IL3) = k(N_T)^2. \qquad (6.3)$$

These equations suggest that some form of cooperation between T cells must occur before lymphokine release can take place. A possible mechanism for this

cooperation is given by the following reaction scheme;

$$T + T + \text{mitogen} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 2T_m$$

where T is a single T cell, and $2T_m$ is a mitogen induced T-T cell conjugate. Equation (6.3) follows if it is assumed that only T-T cell conjugates produce IL3.

One prediction of this model is that presentation of the mitogen on another cell will bypass the requirement for T-T interaction and produce a T cell dose response curve similar to that observed for antigen stimulation. To test this, EL4 tumour cells, which are syngeneic and therefore non-stimulatory, were preincubated with Con A or $NaIO_4$ prior to being added to the reaction wells at 2.5×10^5 cells/well. Figures 6.3 and 6.4 show that the linear section of the plot of $\log_{10}$ (IL3) versus log N_T changed in slope from 2 to 1. Thus, incubation of cells with mitogens renders them similar to an antigenic cell.

6.3.2 Con A presented on a solid substrate is non-stimulatory

One explanation for the requirement for cellular presentation of the lectins is that they bind to the T cell receptor and that lymphokine triggering requires receptor cross linking which is provided in this case by the surface

of another cell. To test this, Con A covalently bound to Sepharose was used to trigger IL3 release. The T cell dose response, shown in Figure 6.5, reveals that IL3 triggering by this agent only occurs at high T cell doses and that the relationship between T cell dose and IL3 titre is of an even higher order than that obtained for soluble Con A. This result is inconsistent with the linear relationship expected if the T cells simply bound to and were triggered by this mitogen.

To test whether the inability of surface bound Con A to trigger IL3 production was due to an inappropriate Con A density on the surface of the solid matrix, solutions of Con A at varying concentrations were preincubated in the wells of 96 well microtitre trays for 1 hour prior to addition of activated T cells. To eliminate any effect of "leaking" Con A, T cells were added at a concentration which does not allow sufficient T cell interaction for detectable IL3 triggering in the presence of soluble Con A, but is sufficient for high levels of IL3 to be produced when the lectin is effectively presented on another cell (10^4 T cells/well, as shown in Figure 6.3). Cell binding was determined in control wells which, after incubation with T cells, were subjected to a vigorous washing procedure. The cells which remained were detected using the colorimetric reagent for the cell enzyme hexosaminidase. Figure 6.6 shows that despite significant cell binding there was no evidence that Con A presented in this way could trigger IL3

release. Thus, if cross-linking is required for lectin stimulation it was not reproduced on a solid matrix.

6.3.3 An MHC-negative cell can present Con A to activated T cells

The MHC-negative human tumour cell K562 was used to explore the role of MHC encoded glycoproteins in lectin induced lymphokine triggering. An aliquot of 2×10^5 K562 cells, which had been preincubated with Con A (section 6.2.3) was added to 10^4 activated T cells. Table 6.1 shows that Con A coated K562 triggered equivalent quantities of IL3 compared to the control cell EL4.

6.4 DISCUSSION

The T cell dose response curves presented in Figure 6.1 and 6.2 indicate that some form of T cell cooperation occurs between T cells before lymphokine release is triggered. Evidence that these dose response characteristics are created by a requirement for cell interaction was supplied by adding mitogen pretreated cells to the T cells. These cells behaved as though they were antigenic, yielding a T cell dose response curve with a linear section on a log-log plot with a slope of 1. Thus, in this region a direct relationship exists between T cell dose and IL3 titre. This result indicates that the requirement for some form of T-T cooperation was not obligatory but simply reflects a need for cellular presentation of the mitogen. It is interesting also to note the distinction in T cell dose response curves generated by 0.5 and 5 µg/ml Con A (Figure 6.1). The higher lectin dose will trigger IL3 at T cell concentrations where the lower dose had fallen off to produce a plateau. This result can be explained by considering the reversible nature of lectin binding. The more T cells (i.e. the more lectin binding sites) present in the well the less Con A will be bound per cell. Presumably with increasing T cell doses a level of bound lectin will be reached where the ability to trigger lymphokine release from a T cell will be diminished.

It is also noteworthy that the IL3 titre produced

following lectin stimulation for a given T cell dose was approximately 10-fold higher than that obtained for $NaIO_4$ stimulation, suggesting that either 10-fold less cells are triggered by $NaIO_4$ or that this mitogen triggers IL3 release at a slower rate than lectin stimulation.

The requirement for cellular cooperation for $NaIO_4$ triggering suggests that cells which are mildly oxidized 'appear' antigenic to syngeneic T cells. This response may be analogous to an alloreaction where small changes in the chemical structure of the MHC antigens are capable of being recognized by a large number of T cells (Blanden et al, 1976). This result is consistent with a previous observation that γ-irradiated $NaIO_4$ treated spleen cells could activate a high proportion of syngeneic untreated spleen cells (Greineder and Rosenthal, 1975; McLain et al, 1975).

The requirement for cellular presentation of lectins is more surprising. One possibility is that the lectins bind directly to the T cell receptor and that some form of receptor cross-linking is required before the antigen signal is triggered. This hypothesis is supported by the work of Chilson et al (1984) who have provided direct evidence for binding of mitogenic lectins to the T cell receptor.
In section 6.3.2 no evidence could be found that cross-linking through a solid support is capable of triggering IL3

release. The unusual dose response obtained for Con A-Sepharose stimulation could be explained if Con A is removed from the beads at a rate dependent on the T cell number and that the soluble Con A and T cells then cooperate to effect lymphokine release. These observations are interesting if considered in terms of the drug receptor theory used in the study of phamacology. Calling the T cell receptor, R, yields the following reaction,

$$R + \text{Con A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{R-Con A} \xrightarrow{k_3} S$$

where S is the antigen signal, k_1 and k_2 are rate constants and k_3 is the intrinsic activity term which indicates the efficiency with which the R-Con A complex mediates the antigen signal. Three experimental conditions have been used which could generate R-Con A complexes on the T cell surface; Con A presented in a soluble form, Con A presented on the surface of a cell and Con A attached to a solid matrix. Only when Con A is bound to a cell is the T cell signal generated. Equating Con A with the specific case of antigen stimulation suggests that soluble antigen or antigen bound to a solid matrix, such as might occur on the tegument of a nematode parasite, cannot trigger the T cell antigen signal even if they could bind to the receptor. Thus the intrinsic activity requirements of signal generation, could confine T cell reactivity to cell-associated antigens.

The above discussion applies only if lectins act by binding to the T cell receptor. It is also possible that

they act in the manner already suggested for $NaIO_4$ and alter in some way the cell surface. Thus, triggering by lectins would involve 'specific' recognition via the T cell receptor and not bridging between receptor and ligand. This model has been proposed previously by Kimura and Ersson (1981) to account for their finding that all mitogenic and no non-mitogenic lectins bind to MHC glycoproteins. However, it is difficult to imagine how nearly 100% of all T cells could be stimulated by Con A (Chen et al, 1982) if it acted simply by altering the antigenic appearance of the cell. It seems more likely that Con A triggers the T cell by its capacity to non-specifically bind to sugars carried by the T cell receptor (Chilson et al, 1984; Kanellopoulis et al, 1975) and that the observed correlation of mitogenic lectins with MHC binding (Kimura and Ersson, 1984) indicates some other activation requirement. One possibility is the release of IL1 from accessory cells in culture. Lafferty has suggested that the MHC products on these cells control release of IL1 from S^+ accessory cells (Lafferty et al, 1983). Thus, for a lectin to be mitogenic for resting T cells, which require both antigen and IL1 signals (Larsson et al, 1979), it must be capable of binding to the T cell receptor and to the MHC. If this were true, triggering of lymphokine release from activated T cells, a reaction which has no accessory cell requirement, would not require the lectin to bind to the MHC. Evidence in support of this hypothesis was presented in section 6.3.3 which showed that the human cell line K562, which does not possess detectable MHC antigens (Klein et al, 1980) can

present Con A in a stimulatory form for lymphokine release. A contradiction however, is that mitogenic lectins do not trigger IL1 release from purified S^+ cells (Andrus, 1981). This could be explained by assuming that the MHC products on the S^+ cell must be cross-linked in a manner most efficiently performed by the T lymphocyte.

To summarize this view, lectin stimulation actually occurs by a mechanism which is similar to the steps required for alloantigen stimulation. In the first reaction, the activation step, the resting T cell has its antigen receptors cross-linked after contact with lectin coated cells (not necessarily S^+ cells). Lectin-coated S^+ cells are triggered through their cell surface MHC to release IL1 following interaction with T lymphocytes. This product completes the activation requirements for the lectin triggered lymphocytes. The activated T cells then interact with other lectin-coated cells in culture (any cell) and are triggered to release lymphokines which include IL2. The activated T cells then proliferate in response to this factor.

TABLE 6.1

The K562 cell line can stimulate IL3 release when coated with Con A

Treatment	IL3 activity (Log_{10} endpoint titre)
T only[a]	≤0.3
T + Con A[b]	≤0.3
EL4 only[c]	≤0.3
K562 only	≤0.3
T + EL4	≤0.3
T + K562	≤0.3
T + EL4 + Con A	1.90 ± 0.1[d]
T + K562 + Con A	2.36 ± 0.09

a) 10^4 seven day-activated B6 anti-BALB/c T cells in 200 µl EMEM + 10^{-4}M 2-ME. Assays performed as described in section 6.2.1.

b) 5 µg/ml Con A

c) Each well received 2 x 10^5 EL4 or K562.

d) Mean ± 95% confidence interval.

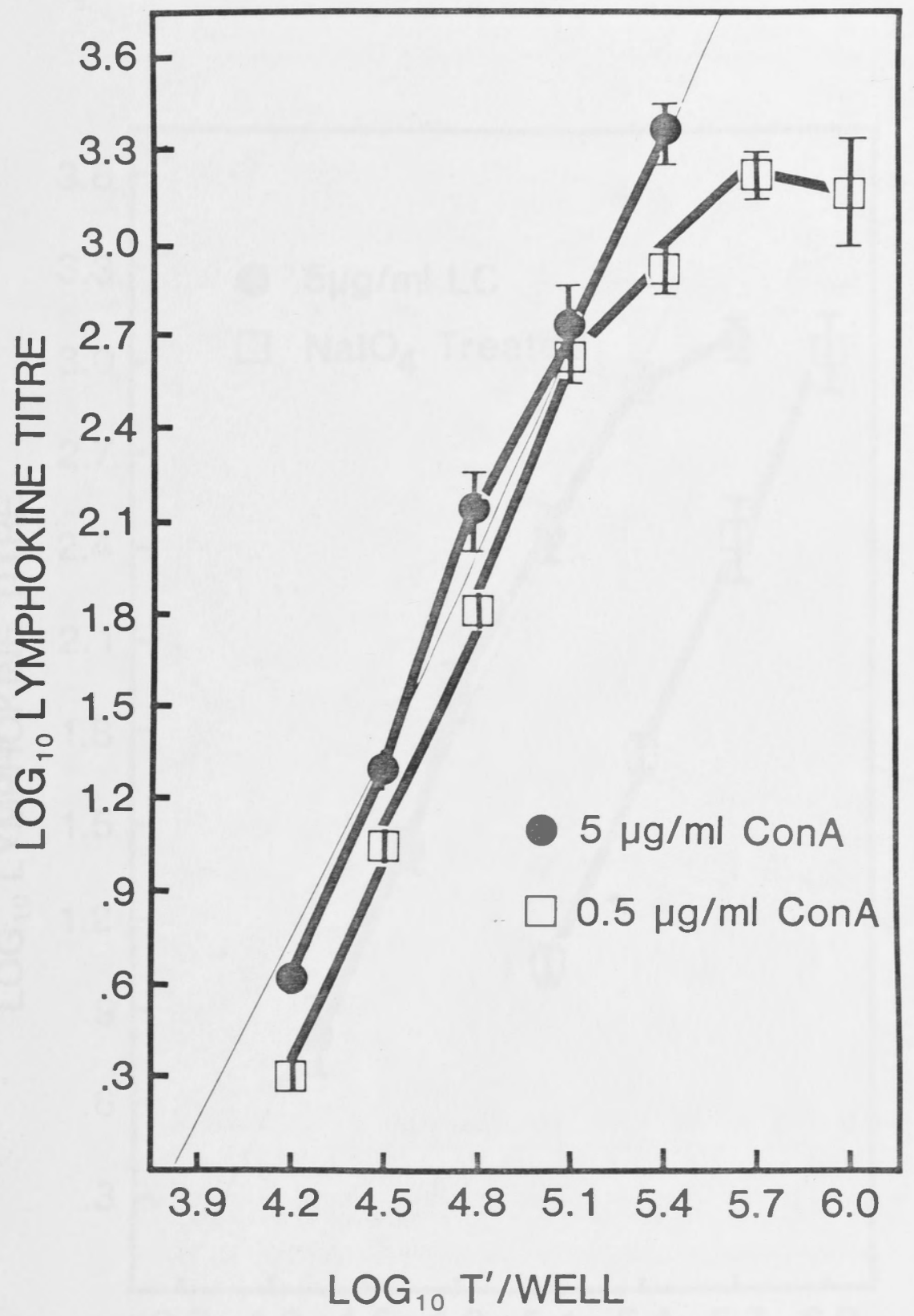


Figure 6.1. The T cell dose response curve generated using two concentrations of Con A. Seven day-activated B6 anti-BALB/c T cells (T') were incubated at varying numbers with 5 or 0.5 µg/ml Con A. At the end of 6 hours the supernatants were collected and assayed for IL3 activity. Each point is the mean plus 95% confidence intervals calculated from four replicates. The thin line is included as a reference and represents a slope of 2.

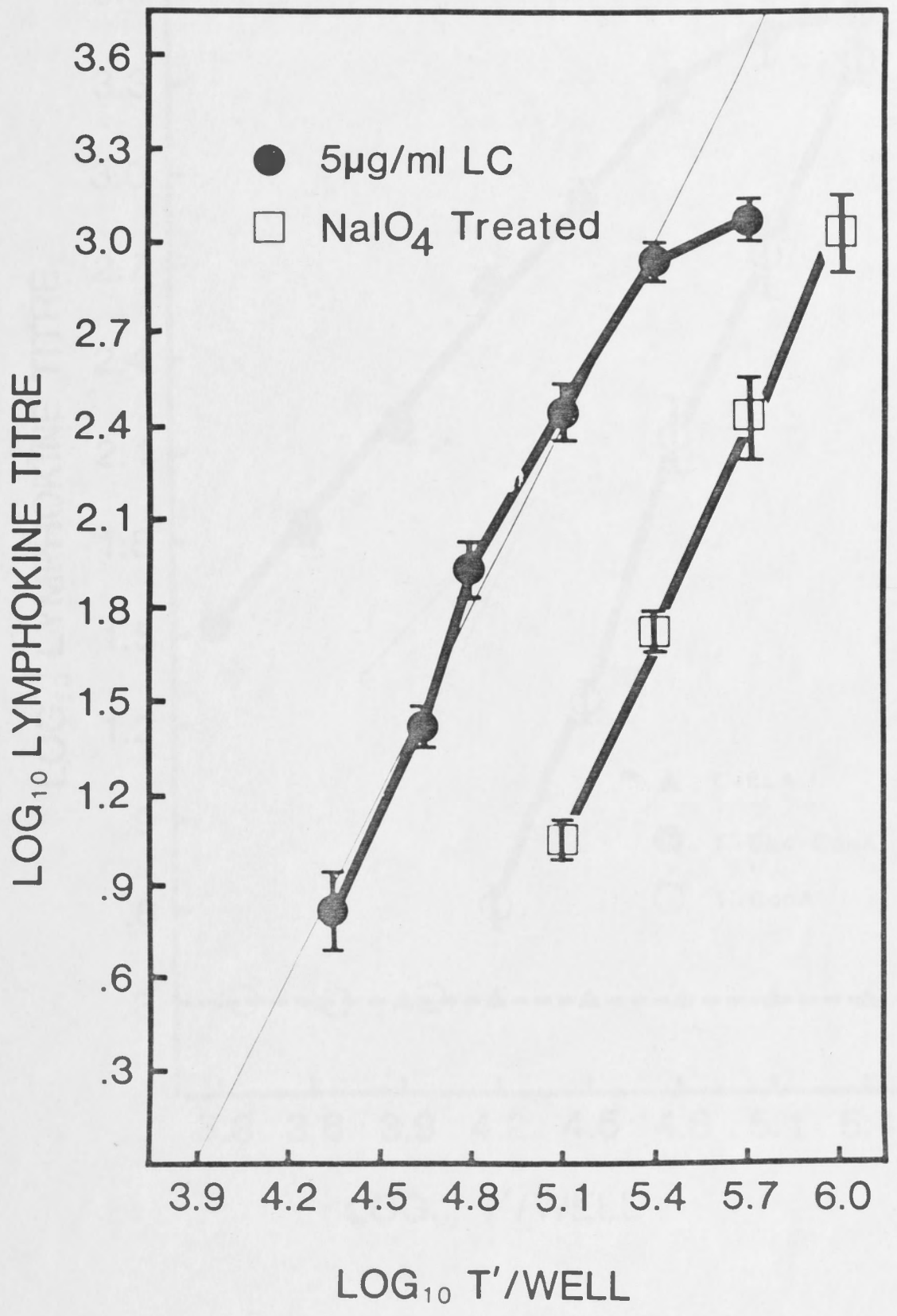


Figure 6.2. T cell dose response after stimulation with leucoagglutinin (LC) or $NaIO_4$. Seven day-activated B6 anti-BALB/c T cells (T') were incubated with 5 µg/ml leucoagglutinin for 6 hours, or were treated with 10^{-4}M $NaIO_4$ prior to being placed in culture. Each point is the mean and 95% confidence interval calculated from four replicates. The thin line represents a slope of 2.

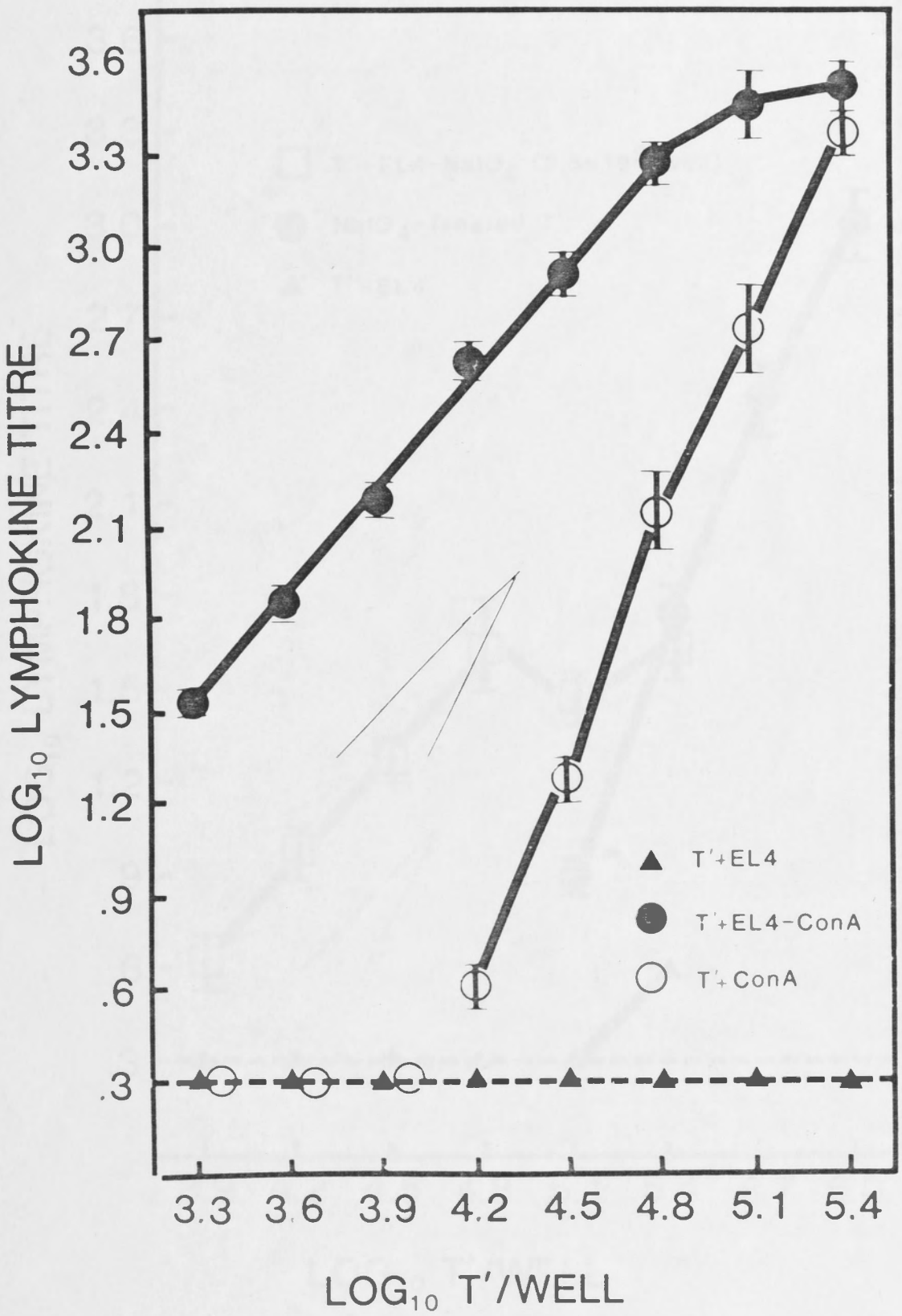


Figure 6.3. T cell dose response curves. Varying doses of seven day-activated T cells were incubated with 5 µg/ml Con A, 2.5 x 10^5 UV-irradiated EL4 or 2.5 x 10^5 UV-irradiated EL4 which had been previously incubated for 15 min with 15 µg/ml Con A (EL4-Con A). Supernatants were collected after 6 hours and assayed for IL3. Each point is the mean ± 95% confidence intervals calculated from four replicates. The thin lines are included for reference and represent slopes of 1 and 2.

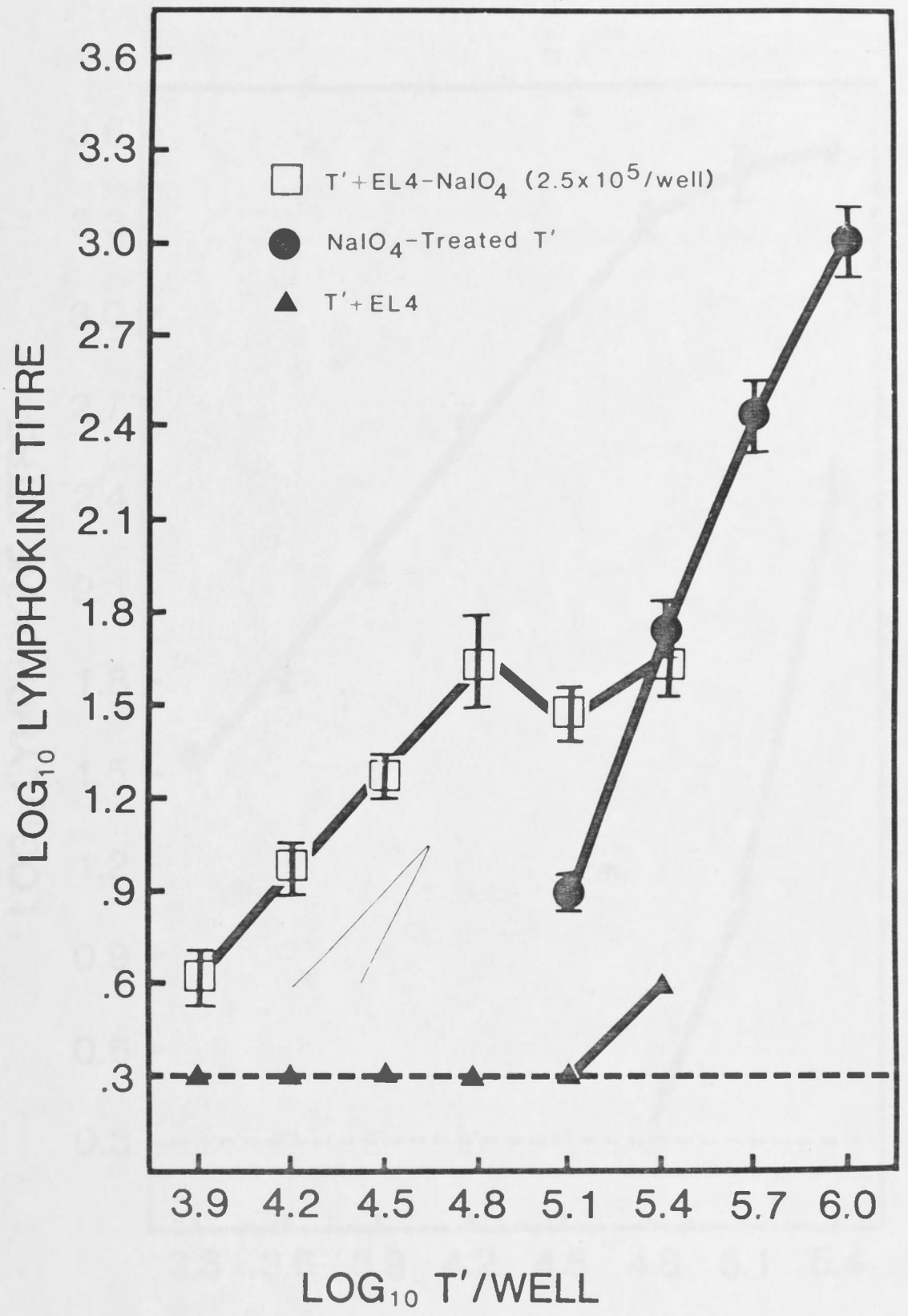


Figure 6.4. T cell dose response curves. Varying doses of activated T cells were incubated with either; 2.5 x 10^5 UV-irradiated EL4 or 2.5 x 10^5 UV-EL4 which had been treated previously with 10^{-4}M $NaIO_4$ for 10 min ($NaIO_4$-EL4). Also shown is the response obtained for $NaIO_4$-treated T cells only. Supernatants were harvested after 6 hours and assayed for IL3 activity. Each point is the mean and 95% confidence intervals based on four replicates. The thin lines are included for reference, and depict slopes of 1 and 2.

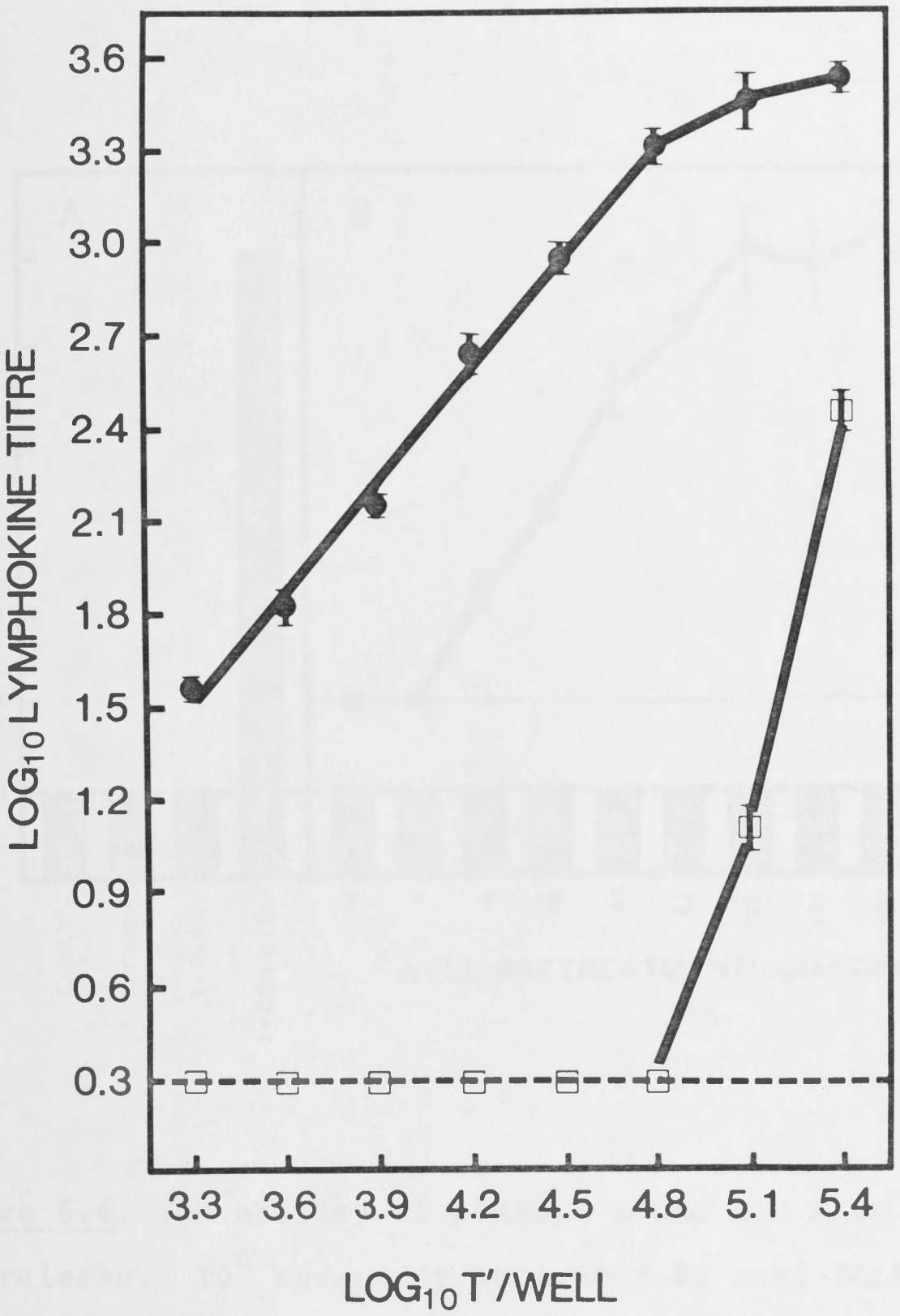


Figure 6.5. Con A-Sepharose stimulation of lymphokine release. Varying numbers of activated T cells were incubated with 0.2 mg/ml gel sediment of a commercial preparation of Con A-Sepharose (Pharmacia). The IL3 titres obtained after 6 hours incubation are represented by the open squares. The closed circles are the response obtained from incubating the same T cell numbers with Con A pretreated EL4 (from figure 6.3). Each point is the mean and 95% confidence limits based on 4 replicates.

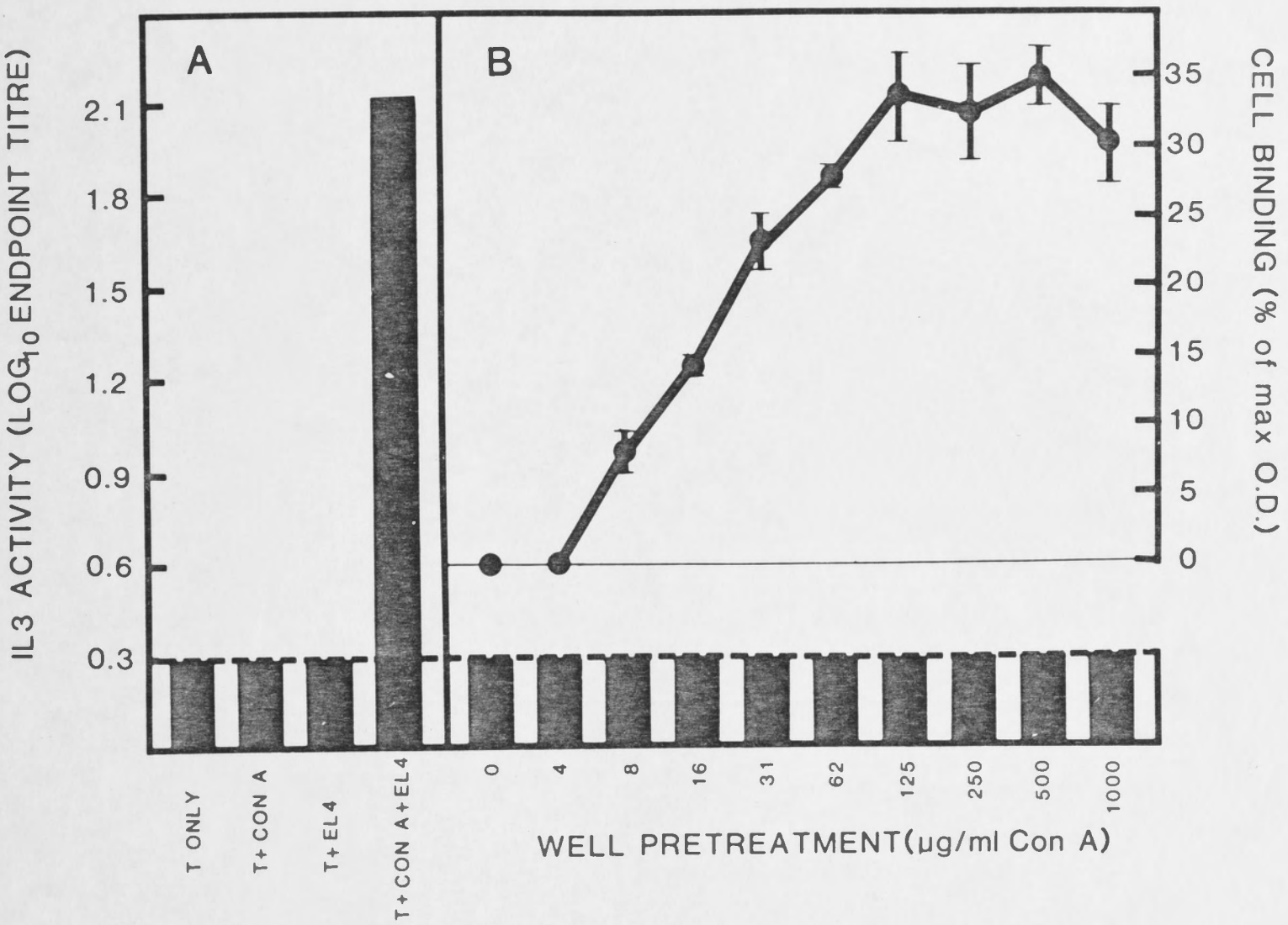


Figure 6.6. The ability of plastic-bound Con A to trigger IL3 release. 10^4 seven day activated B6 anti-BALB/c T cells were incubated for 6 hours in wells of a 96 well microtitre tray. In the control wells shown in panel A, T cells were incubated either alone, with 5 µg/ml Con A, with 2.5 x 10^5 UV-irradiated EL4 or with both Con A and EL4. In panel B the wells were preincubated for 1 hour at 37°C with the concentrations of Con A shown, before 10^4 T cells were added and incubated a further 6 hours. The level of T cell binding to these Con A coated wells was determined in a group of control wells as described in section 6.2.4.

CHAPTER 7

A QUANTITATIVE ANALYSIS OF CELL DOSE RESPONSE CURVES FOR LYMPHOKINE RELEASE

7.1 INTRODUCTION

In chapter 3 it was concluded from an analysis of T cell dose response curves, that the triggering of IL3 release from activated T cells is dependent solely upon T cell-target cell interaction. In this chapter a more complete examination of the relationship between cell dose and lymphokine titre was attempted. It was found that the dose response curves of lymphokine titre versus cell number (either T cell or antigenic cell) behave in quite complex ways which are not readily explained by simple one to one binding between the T cell and the target cell. A mathematical analysis of this system was attempted by first determining what set of basic assumptions about T cell responsiveness and in vitro cell interaction can adequately account for the observed experimental results.

Two points were of particular interest. Firstly, whether T cell triggering is an all or none response, or a graded response. That is, does a single T cell, when triggered, release a constant amount of lymphokine characteristic of that particular T cell and independent of the strength of the triggering signal or, alternatively, is the amount of lymphokine released by an individual cell dependent on the nature of the triggering stimulus. The second point is whether or not a single T cell-target cell interaction is a sufficient stimulus to initiate lymphokine release or whether multiple cellular interactions are necessary. The

results reported in this chapter are consistent with the hypothesis that T cell triggering is an all or none event and that binding of at least two antigenic cells to a single T cell is necessary to trigger lymphokine production in that cell.

7.2 MATERIALS AND METHODS

7.2.1 IL3 release assay

Seven day-activated B6 anti-BALB/c T cells were used in all experiments. Suspensions of T cells and of UV-irradiated P815 tumour cells were prepared in EMEM containing 10^{-4}M 2-ME. An aliquot of 100 µl of T cell suspension was mixed with 100 µl of P815 cells in individual wells of a 96 well microtitre culture tray (NUNC) and incubated at 37°C in a humidified atmosphere of 10% CO_2 in air for 5 hours unless otherwise indicated. After culture the supernatant was removed and stored at -20°C prior to assay for IL3 activity.

7.2.2 IL3 assay

Each IL3 assay was performed using the FD IL3 indicator cell line in the assay procedure described in section 2.12B

7.2.3 ^{51}Cr-labelling P815

Live and UV-irradiated P815 were labelled with ^{51}Cr using the procedure described in section 2.13.

7.3 RESULTS

7.3.1 Experimentally derived cell dose response curves for lymphokine release

In Figure 7.1 the basic relationships between IL3 release and T cell or P815 number are shown. Figure 7.1A is the dose response curve obtained by varying P815 cell number while holding the total T cell number constant. The relationship is linear with an approximate slope of two in the region of T cell excess with a plateau in the region where T cell and P815 cell numbers are comparable. In Figure 7.1B the T cell dose response curve is shown for a constant P815 cell number. The slope of lymphokine titre versus T cell number is close to unity throughout the region of antigenic cell excess. Figure 7.1C shows the dose response curve obtained when both T cell and P815 cell numbers are varied together. The slope of the relationship lies between two and three over the main region of the graph showing a very gradual roll-off at the highest total cell number.

These simple relationships between cell number and lymphokine release effectively delimit the possible characteristics of any model of lymphokine release. The fact that the relationship between antigenic cell number and lymphokine titre has a slope greater than unity indicates that the order of the reaction between T cells and antigenic cells is greater that one, i.e. more than one antigenic cell

must bind to the T cell before lymphokine release can be triggered. That the slope is approximately equal to two suggests that a minimum requirement for triggering of a single T cell is the binding of at least two antigenic cells. That the slope of IL3 titre versus T cell number is approximately equal to one indicates that under the specific conditions of this experiment there is little or no effective cooperation between T cells. The form of the relationship in Figure 7.1A, a linear region at low antigenic cell number asymptoting to a fixed value in the region of antigen excess, is analogous to classical binding curves suggesting that as a first approximation T cell-antigen cell interactions may be treated as simple steady state binding reactions.

7.3.2 A model of T cell-target cell interaction

A basic model relating T cell and antigenic cell number to lymphokine release can now be derived with the use of two assumptions. It is assumed that the rate limiting step of lymphokine production is the intra-cellular process of lymphokine synthesis and release, not the initial inter-cellular triggering events. That is to say, the processes of T cell-antigenic cell recognition, binding and triggering are considered to be fast, compared to the time necessary for a triggered T cell to manufacture and release detectable lymphokine. The second assumption made is that the amount of lymphokine released is directly proportional to the

number of T cells which have bound to at least two P815. The amount of lymphokine produced can therefore be expressed by the following equation.

$$[LK] = aN_{TA_2} \qquad (1)$$

where, [LK] is the concentration of lymphokine in the supernatant, N_{TA_2} is the total number of T cells bound to two antigenic cells and a is a proportionality constant.

With these two assumptions the simplest binding model which is consistent with the results of Figure 1. is as follows.

$$T + A \overset{K}{\longleftrightarrow} TA + A \overset{K}{\longleftrightarrow} TA_2 \overset{\alpha}{\longrightarrow} [LK]$$

where, T is a T cell unbound to any antigenic cells, TA is a T cell bound to a single antigenic cell, TA_2 is a T cell bound to two antigenic cells and α is the proportion of T cells bound to two antigen cells which are triggered to produce lymphokine. This model predicts that the amount of lymphokine released by a given number of T and antigen cells can be described by the following equation.

$$[LK] = a\, N_{TA_2} = N_{T_{(tot)}} \Big/ \left(1 + \frac{2K}{N_A} + \frac{(K)^2}{(N_A)} \right) \qquad (2)$$

where, N_T is the total T cell number per well and N_A is

the number of antigenic cells unbound to any T cell. Derivation of this equation is given in Appendix 7.1

Figure 7.2A shows the family of curves predicted by the steady state binding model relating antigen cell number to lymphokine release for different values of total T cell number. The main predictions of the model are that the linear region of the dose response relationship will have a slope of two and that this region of the curve will gradually merge in assays at high T cell concentrations. The model also predicts that the difference between asymptote values of lymphokine production should remain directly proportional to the total T cell number per well. Figures 7.2B and 7.2C show experimental results with fitted curves. There is clearly good agreement between the predictions of the theory and the experimental results.

Figures 7.1B and 7.3 show T cell titration results with curves fitted using the steady state binding model. The model predicts an initial linear region of slope approximately equal to one, a short plateau region and then a decline in lymphokine production with increasing T cell number. The explanation for the decline of lymphokine production at high T cell numbers is that at the highest T cell concentrations competition between T cells for binding of target cells becomes significant and the number of T cells bound to two cells actually falls.

The prediction of the steady state binding model for the case when T cell and antigen cell numbers are increased simultaneously is quite complicated. The model predicts an initial region with a slope of three, then a transitional region with slope gradually changing from three to one and a final region of slope equal to one. The practical experimental range is relatively restricted and in this region the slope of lymphokine titre versus total cell number is less than three and greater than one as is shown in Figure 7.1C.

7.3.3 The antigen dose response curve generated at different incubation times

Figure 7.4 shows the behaviour of the P815 dose response curve at a number of different times after the initiation of culture. A feature of these curves is that the linear section does not alter between 8 and 16 hours whereas the plateau section continues to increase. This suggests that T cells can only release a certain quantity of IL3 before they must be retriggered by another target cell interaction. In the linear section, retriggering is unlikely as most target cells will have been bound. In target cell excess retriggering can occur. This phenomenon could be due to the lysis of the target cells following contact with T cells. As the target cells were UV-irradiated it was of interest to test their stability with and without added T cells.

Figure 7.5 shows that the rate of ^{51}Cr release from live and UV-irradiated P815 was similar for approximately 4 hours. However, by 16 hours most irradiated cells had released their radio-label. When incubated with an excess of T cells, both live and UV-irradiated P815 released most of the available ^{51}Cr within 3 hours. Thus, in the lymphokine release cultures with T cells in excess, most target cells will have lysed within 3 hours.

7.4 DISCUSSION

The rationale for attempting to mathematically model lymphokine production is that it necessarily forces an explicit statement of the basic assumptions about events occurring at the cellular level. It is possible to explain our experimentally derived cell dose response curves using two assumptions. The first assumption is that cell recognition and binding is a relatively fast process in comparison to the overall time course of the experiment and that these processes are in equilibrium. The assumption is supported by Figure 7.4 which shows that the slope of the linear section of the antigen dose response curve is similar at 4, 8 or 16 hours. The second assumption is that T cell triggering is an all or none process. In terms of the steady state binding model this means that T cells which have bound only a single antigen cell simply do not receive sufficient stimulation to trigger lymphokine release, whereas T cells binding two or more antigen cells do receive a threshold stimulus which is necessary to trigger lymphokine production. In this model there is no partial triggering or graded reponse; a T cell can only be triggered or quiescent. This is not to say that all triggered T cells produce the same amount of lymphokine, simply that further stimulation by, for example, the binding of more antigenic cells, does not result in an increase in lymphokine production over that produced by the threshold amount of stimulation.

Figure 7.6 shows the predictions of the steady state

binding model if it is assumed that some proportion of T cells bound to only one antigen cell can also be triggered. For curve a. all T cells bound to only one antigenic cell as well as those bound to two antigen cells were considered to be triggered. The slope of the linear region of the curve is now equal to unity. For curve b. only 10% of the T cells bound to a single antigen cell were triggered. It is clear that even in this limited case, where only a small fraction of T cells bound to a single target cell are triggered, that there is a significant deviation from curve c. the case, where only T cells bound to two target cells are triggered. The system appears to be quite sensitive to variations in the threshold for triggering. While it remains obvious that a T cell can bind to more than two target cells it appears that this is a minimum requirement for triggering in our system.

The finding that the linear region of the dose response curve for P815 cells is equal to two is in one sense simply fortuitous. Other systems which have been studied have slopes closer to unity for antigen cell dose response curves (Sinickas et al, submitted for publication; Lafferty personal communication). This suggests that in these systems the binding of only one antigen cell to the T cell is sufficient stimulus to trigger. A simple explanation of these observations is possible when triggering is discussed at a single cell level. In classical pharmocology the

relationship between receptor, ligand (antigen) and signal is given by the scheme:

$$R + L \underset{k_2}{\overset{k_2}{\leftrightarrow}} R\text{-}L \xrightarrow{k_3} S$$

k_1 and k_2 determine the affinity of binding while k_3 relates the efficiency with which the number of R-L complexes is translated into the signal. For lymphokine triggering it is assumed that the strength of S must reach a threshold level before any reaction occurs. As the T cell can only get discrete quantities of ligand depending on the number of target cells bound, then the threshold signal level must be reached after binding a 'threshold' number of target cells. The observation that anti-viral T cells (Sinickas et al, submitted for publication) or other alloreactive T cells (Lafferty personal communication) reach a target cell threshold at one, whereas for B6 anti-BALB/c plus P815 the threshold is reached at two, can be explained in one of three ways. Either:

1) the number of receptors per T cell is significantly higher in these reactions,

2) The density or total number of antigenic ligand on the target cell is higher, or,

3) k_3 (efficacy or intrinsic activity) is greater for some ligands or some target cells.

It seems unlikely that the T cell receptor number will vary with specificity. Thus, the latter two explanations probably account for the differences in triggering systems.

The modelling approach used is not without limitations. The model does not hold over the extreme range of cell numbers. At the very highest T cell or P815 cell concentrations the amount of lymphokine produced was lower than predicted by the model. This may simply be a result of some relatively non-specific metabolic effect due to cell crowding or it could be due to non-specific binding between like cells. For example, at high P815 concentrations if there was any significant binding between P815 cells this would effectively reduce the number of P815 cells available to bind to T cells and result in a concomitant reduction in lymphokine titre. It is also true that the assumptions upon which the model is based are not the only ones which could be used to build a model. As is true of any binding or kinetic study, other than the most simple case, a number of different models will give predictions which are indistinguishable within experimental error. It is to be hoped however, that by attempting to quantify the relationships controlling lymphokine production a more accurate description of the processes occurring at the cellular level will ultimately result.

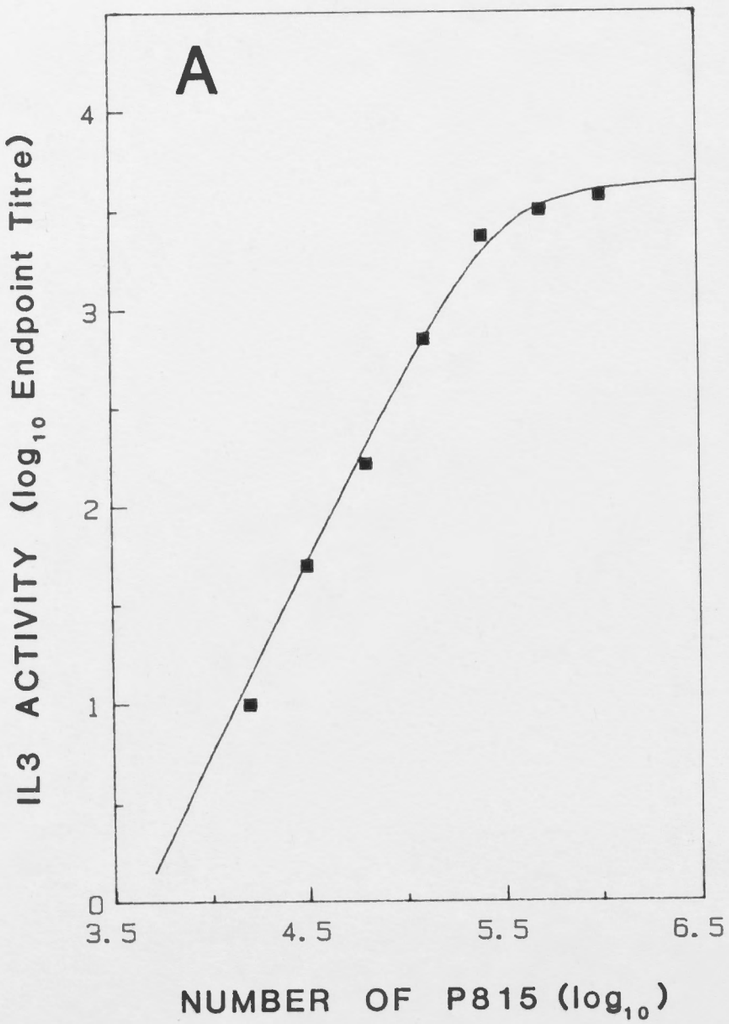
Figure 7.1

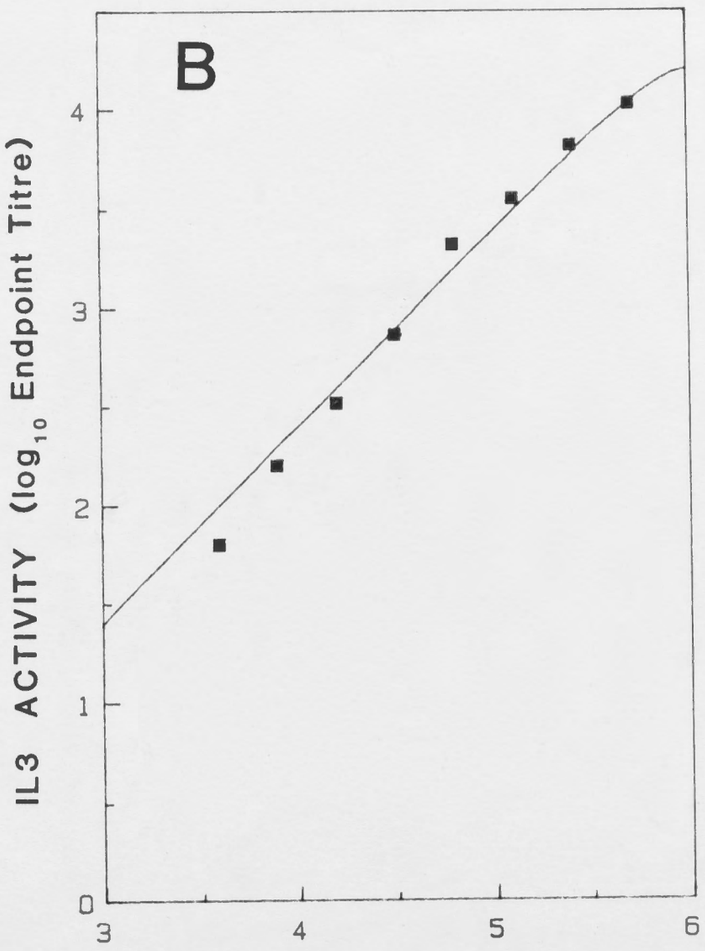
A) Antigenic cell dose response curve. Varying numbers of UV-irradiated P815 were incubated with 2 x 10^5 B6 anti-BALB/c seven day-activated T cells for 5 hours in a final volume of 200 μl EMEM containing 10^{-4}M 2-ME. Four replicate supernatants for each data point were assayed for IL3 activity.

B) T cell dose response curve. Varying numbers of B6 anti-BALB/c seven day-activated T cells were incubated with 10^6 UV-irradiated P815 for 5 hours in a final volume of 200 μl. Each data point is the mean IL3 titre of four replicates.

C) Combined T cell and P815 cell dose response curve. Equal numbers of UV-irradiated P815 and B6 anti-BALB/c seven day-activated T cells were incubated at different total cell numbers in 200 μl medium for 5 hours.

Data points for these curves were fitted using equation A.9 and substituting for: A, K = 5 x 10^4, tf = 0.6; B, K = 10^5, tf = 0.5; C, K = 10^5, tf = 0.4.


A
IL3 ACTIVITY (log10 Endpoint Titre)
0
1
2
3
4
3.5
4.5
5.5
6.5
NUMBER OF P815 (log10)


B
IL3 ACTIVITY (log10 Endpoint Titre)
0
1
2
3
4
3
4
5
6
NUMBER OF T CELLS (log10)

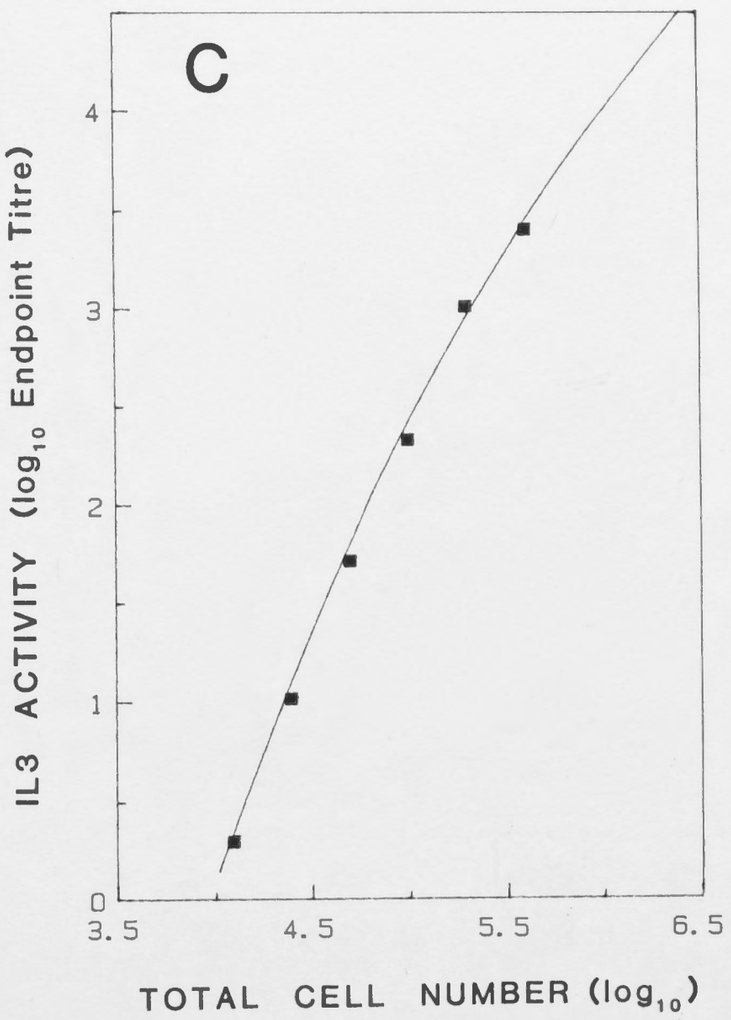
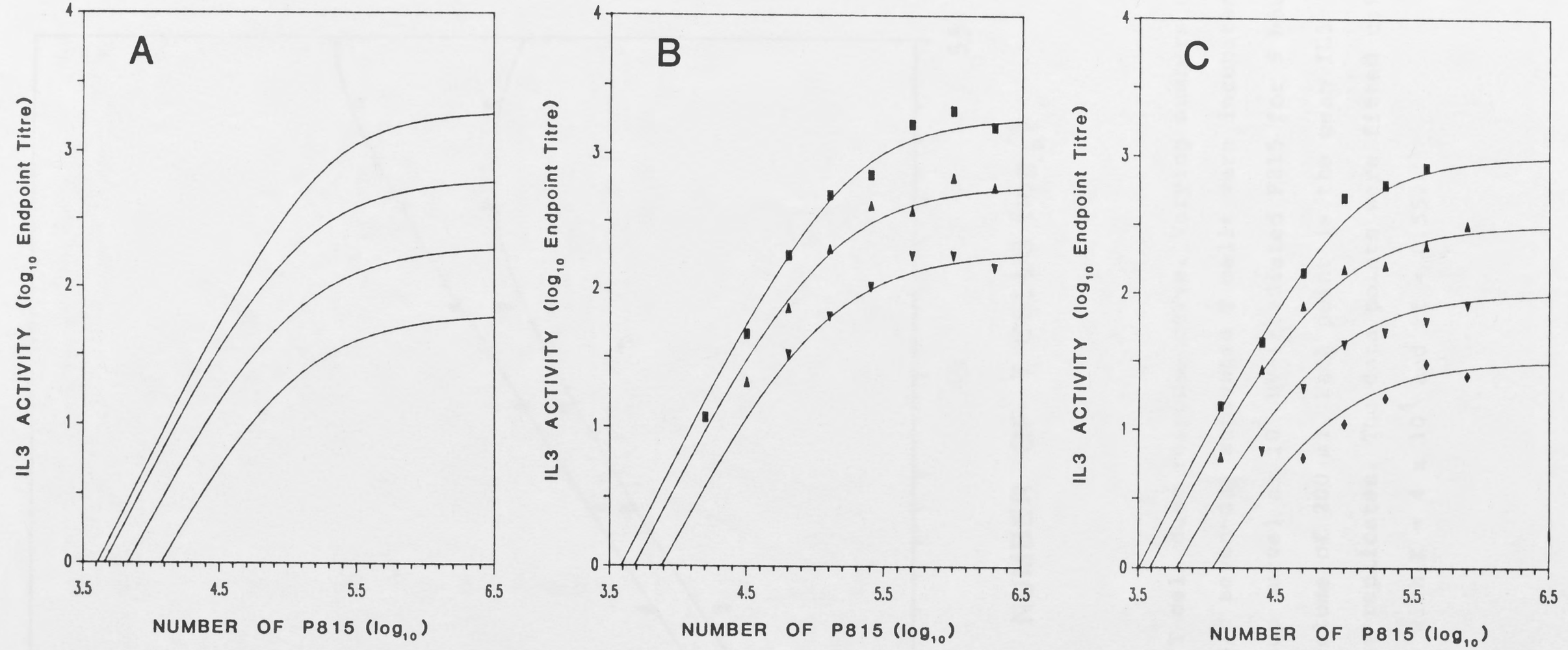

C
IL3 ACTIVITY (log10 Endpoint Titre)
0
1
2
3
4
3.5
4.5
5.5
6.5
TOTAL CELL NUMBER (log10)

Figure 7.2

A) Simulated family of antigen dose response curves with T cell number equal to 10^5, $10^{4.5}$, $10^{4.0}$ and $10^{3.5}$, using equation A.9 with $K = 8 \times 10^4$ and tf = 0.5.

B) Family of experimental P815 dose response curves (1). Varying numbers of UV-irradiated P815 were incubated with (top curve) $10^{4.7}$, $10^{4.2}$, and (bottom curve) $10^{3.7}$, B6 anti-BALB/c activated T cells in a fixed volume of 200 μl. The data points were fitted using equation A.9 with $K = 5 \times 10^4$ and tf = 0.25.

C) Family of experimental P815 dose response curves (2). Same as (B) using T cell numbers (top to bottom) 10^5, $10^{4.5}$, 10^4, and $10^{3.5}$. Fitted parameters were $K = 9 \times 10^4$ and tf = 0.75.


A
B
C
IL3 ACTIVITY (log10 Endpoint Titre)
NUMBER OF P815 (log10)
0
1
2
3
4
3.5
4.5
5.5
6.5

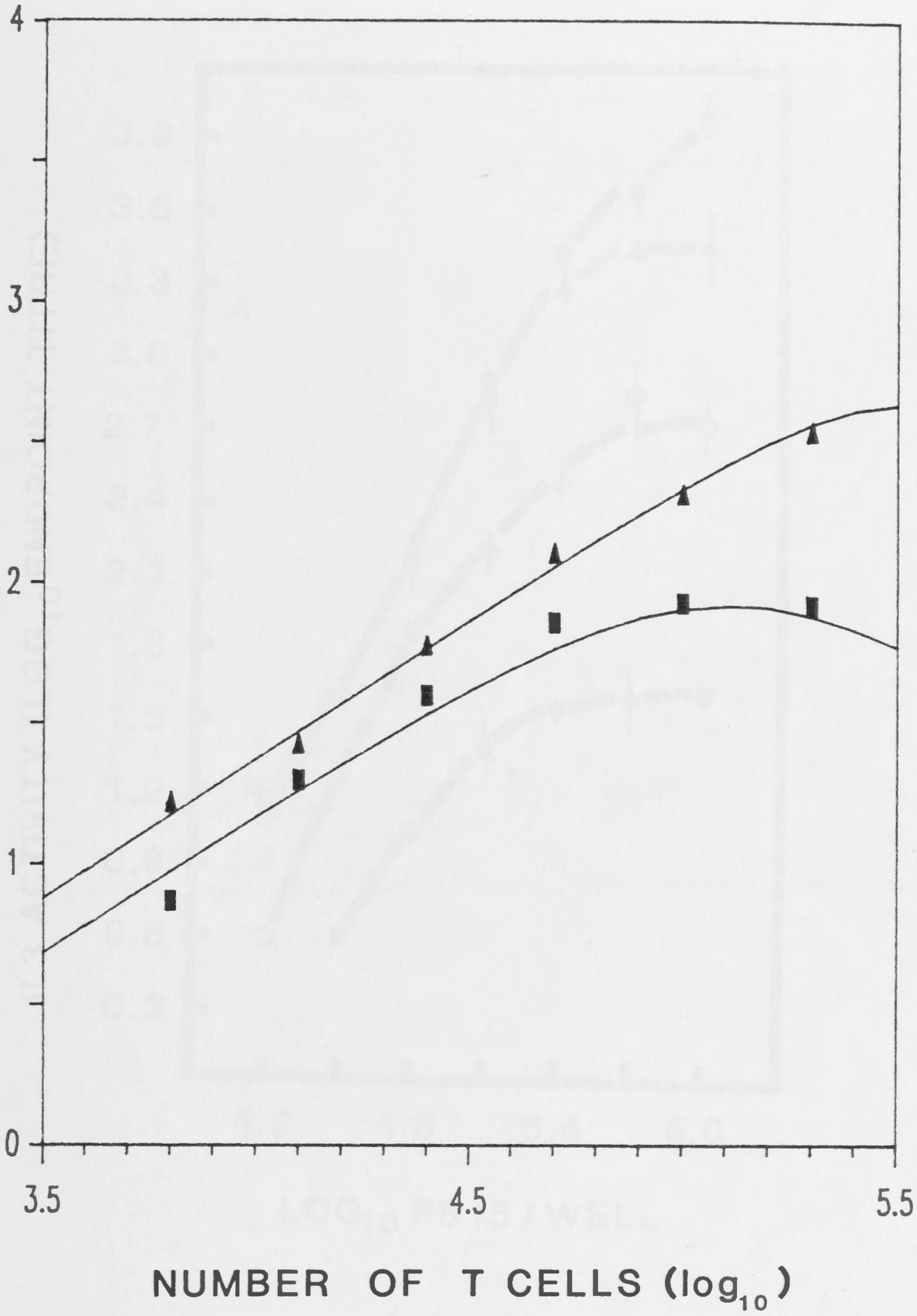


Figure 7.3. T cell dose response curve. Varying numbers of B6 anti-BALB/c seven-day activated T cells were incubated with $10^{5.5}$ (top curve) or 10^{5} UV-irradiated P815 for 5 hours in a final volume of 200 μl. Each point is the mean IL3 titre of four replicates. The data points were fitted using equation A.9 with K = 4×10^{4} and tf = 0.55.

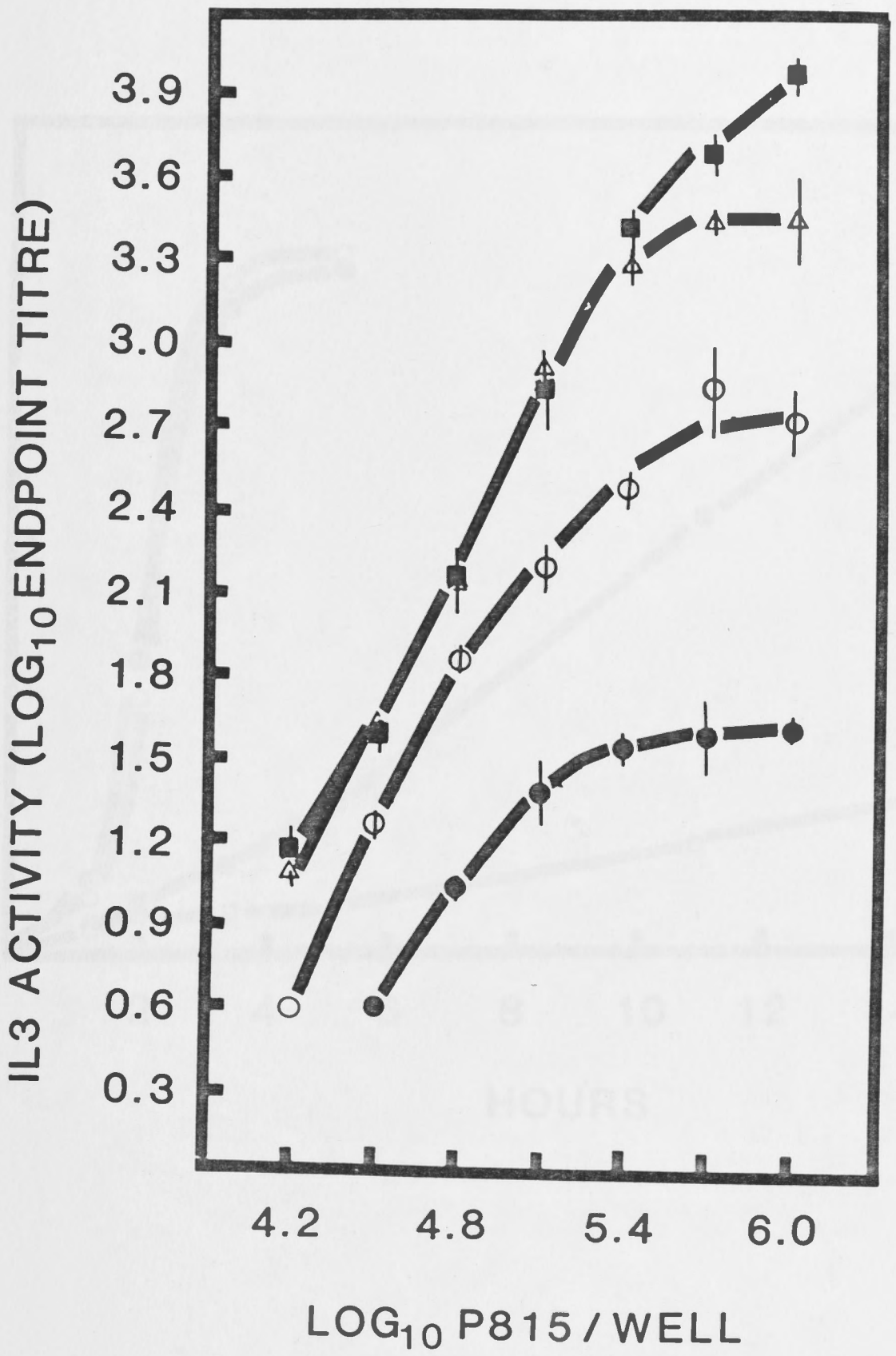


Figure 7.4. P815 dose response curves generated at different times. Varying numbers of UV-irradiated P815 were incubated with 10^5 B6 anti-BALB/c seven day activated T cells for 2 (●), 4 (○), 8 (△) or 16 hours (■). Each point is the mean and 95 % confidence interval of IL3 titres determined from four replicates.

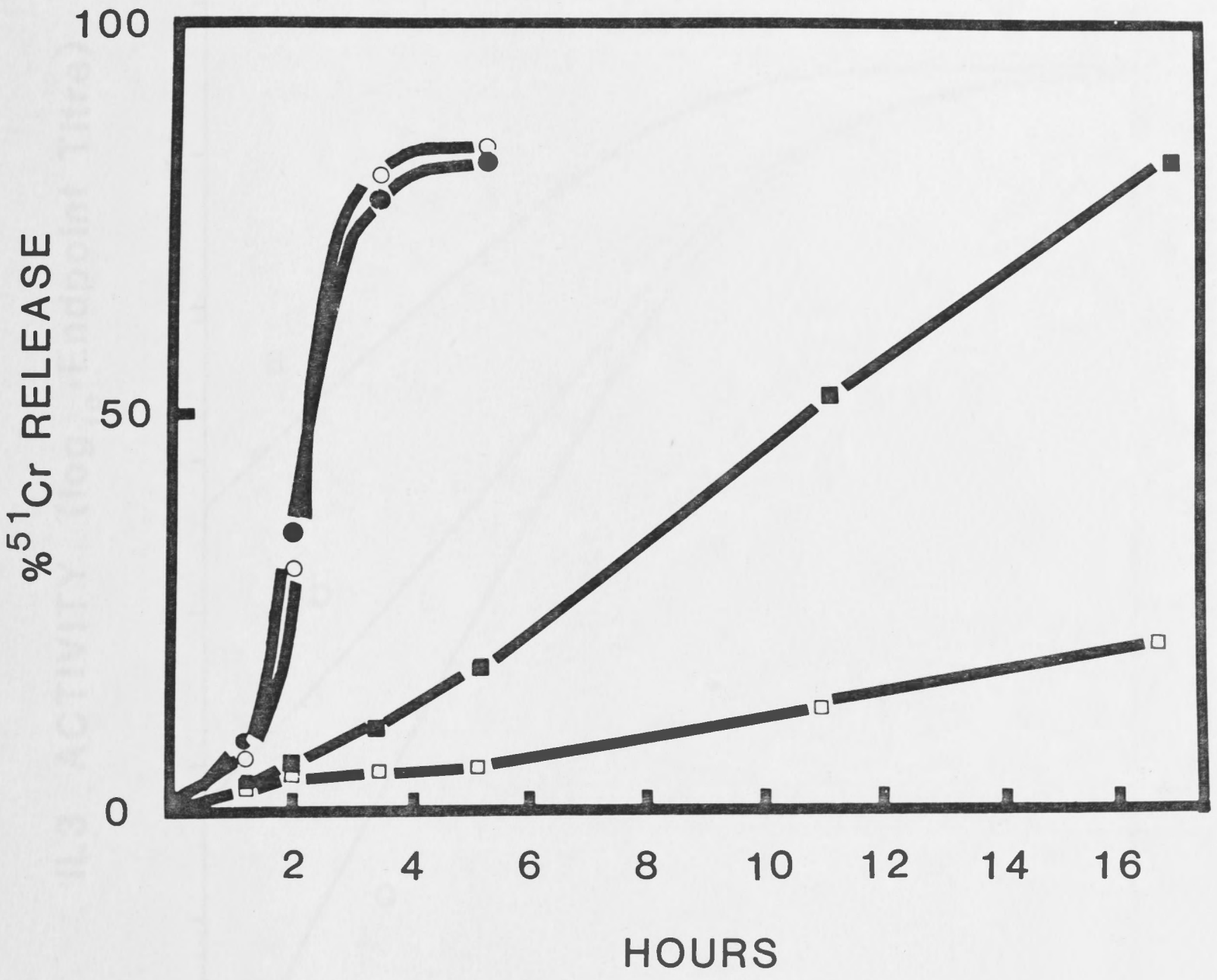


Figure 7.5. ^{51}Cr release from UV-irradiated P815. 2×10^4 live (open symbols) or UV-irradiated (closed symbols) P815 were incubated either alone (squares) or with 10^5 seven day-activated B6 anti-BALB/c T cells in 200 μl. At various times supernatants were removed from triplicate cultures and the level of γ-irradiation measured. 100% release was measured from distilled water treated target cells obtained after a four hour incubation.

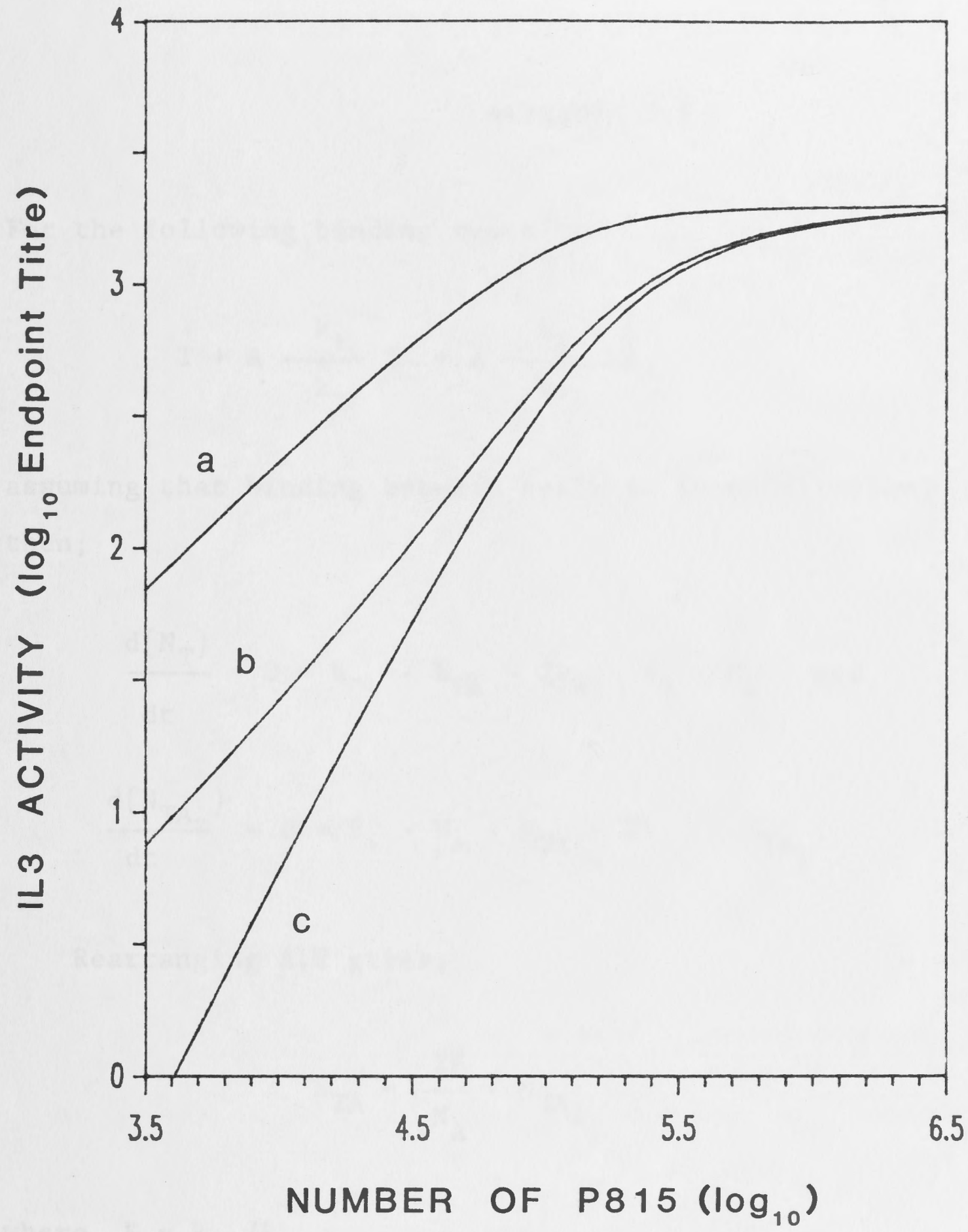


Figure 7.6. Simulated antigenic cell dose response curve with T cell number equal to 10^5, K = 8 x 10^4, and tf = 0.5. Curve (a) represents the situation if T cells release IL3 following binding to 1 or 2 target cells. Curve (b) is obtained if 10% of reactive T cells are triggered following binding to 1 target cell and the other 90% are triggered by binding to 2 targets. Curve (c) is generated if all T cells must bind 2 target cells to trigger IL3 release.

APPENDIX 7.1

For the following binding reaction,

$$T + A \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} TA + A \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} TA_2 ,$$

assuming that binding between cells is in equilibrium, then;

$$\frac{d(N_T)}{dt} = 0 = k_{-1} \cdot N_{TA} - 2k_1 \cdot N_A \cdot N_T \text{ , and,} \qquad (A.1)$$

$$\frac{d(N_{TA_2})}{dt} = 0 = k_1 \cdot N_A \cdot N_{TA} - 2k_{-1} \cdot N_{TA_2} . \qquad (A.2)$$

Rearranging A.2 gives;

$$N_{TA} = \frac{2K}{N_A} \cdot N_{TA_2} \qquad (A.3)$$

where, $K = k_{-1}/k_1$.

Rearranging A.1 and substituting into equation A.3 gives;

$$N_T = \left(\frac{K}{N_A}\right)^2 \cdot N_{TA_2} . \qquad (A.4)$$

Since it is expected that the total number of T cells and antigenic cells will remain constant during the initial binding and triggering period the following relationships will also hold true;

$$N_T + N_{TA} + N_{TA_2} = N_{T_{(tot)}} \qquad (A.5)$$

$$N_A + N_{TA} + 2N_{TA_2} = N_{A_{(tot)}} \qquad (A.6)$$

where, $N_{T_{(tot)}}$ is the total number of T cells per well and $N_{A_{(tot)}}$ is the total number of antigenic cells per well.

Substituting equations (A.3) and (A.4) into (A.5) gives,

$$N_{TA_2} = N_{T_{(tot)}} \Big/ \left(1 + \frac{2K}{N_A} + \frac{(K)^2}{(N_A)^2} \right). \qquad (A.7)$$

Combining equations (A.3),(A.4),(A.5) and (A.6) gives,

$$N_A = \frac{\left(2K\left(N_{TA_2} - N_{T_{(tot)}}\right) - K^2\right) N_{TA_2}}{2KN_{TA_2} - \left(2N_{TA_2} - N_{A_{(tot)}}\right)\left(N_{TA_2} - N_{T_{(tot)}}\right)}. \qquad (A.8)$$

Substituting equation (A.8) into (A.6) and reducing gives;

$$4N_{T_{(tot)}} \cdot (N_{TA_2})^2 - (K^2 + 4(N_{T_{(tot)}})^2 + 4KN_{T_{(tot)}} + 2KN_{A_{(tot)}} + (N_{A_{(tot)}})^2) \cdot N_{TA_2} + (N_{A_{(tot)}})^2 \cdot N_{T_{(tot)}} = 0. \qquad (A.9)$$

To evaluate N_{TA_2} it is necessary to solve the quadratic equation A.9.

To fit the data points it was necessary to take account of the fact that not all of the T cells were primed against the P815 antigen cell. This was achieved by scaling the number of T cells per well by a constant factor 'tf' which was taken to be the proportion of T cells primed against the antigen cell P815. The effective number of T cells per well is then given by,

$$N_{TA_2(effective)} = tf \cdot N_{TA_2} \qquad (A.10)$$

The other free parameters for fitting were K, the apparent dissociation constant and a the scale factor for the average amount of lymphokine released per T cell.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

In this final chapter I propose to discuss some of the more general implications of the work already described.

A 'universal' T cell assay

A variety of procedures and signals have been found to influence lymphokine release. Early work with primary bulk cultures found that release of lymphokines from T cells required accessory cells or IL1 and that peak titres occurred after approximately 16 hours in culture (Andersson et al, 1979; Thoman and Weigle, 1980; Swain and Dutton, 1980). Work with IL2-dependent T cells or T cell hybridomas have revealed a different pattern - usually release is non - IL1 dependent and peak titres occur within 10 hours (Ely et al, 1981; Kelso et al, 1982; Prystowsky et al, 1982; Kelso and Glasebrook, 1984; Andrus et al, 1984). Shimonkevitz et al (1983) showed that release of IL2 from an ovalbumin-specific T cell hybridoma required an accessory cell, however, the function of this cell was solely to present antigen since fixation with glutaraldehyde did not inhibit the stimulating function of the cell. Class 2 MHC-bearing 'accessory cells' are an obvious requirement for class 2-MHC resticted T cell responses. The different requirements for triggering lymphokine release from resting and activated T cells could simply be due to the need for activation of the resting cell, with subsequent lymphokine triggering being mediated by the same reaction as occurs for IL2 dependent-T cells. This would explain the requirement for IL1 and the longer time required to achieve maximal lymphokine release.

As there are many different lymphokines with diverse functions it is clear that the role a T cell will play in an immune reaction is to a large degree determined by the lymphokines it will produce. An important question then is what controls the release of lymphokines and what influences the type made by a particular cell.

In chapter 3 a study of the reproducibility, dose response, and time course behaviour of both IL2 and IL3 release from in vitro activated T cells reached the following conclusions: the release of both lymphokines is linked, exposure to antigen alone is a sufficient stimulus to induce the release of both lymphokines and the triggering process requires synthesis of lymphokine-encoding mRNA. Kelso and Glasebrook (1984) examined the behaviour of a large number of T cell clones and found that most cells release some lymphokine activity although the amount and the range produced by a given cell is unpredictable. Individual clones, however, make the same lymphokines in approximately equivalent amounts after each antigen exposure. A general conclusion from this work and the work described in chapter 3 is that activated T cells have a 'repertoire' of lymphokines whose release is dependent on the antigen signal. Each individual cell has a characteristic repertoire but the signal which controls the release of lymphokines is common to all T cells. This implies that measurement of lymphokine release can be related to triggering of the antigen signal and that the release

characteristics of a single lymphokine will be representative of the entire lymphokine repertoire. This discussion illustates the rationale for the use, in this thesis, of a single lymphokine release assay (IL3 release) to draw general conclusions about the T cell antigen signal.

Other experimental procedures have shown an effect of non-antigen signals on lymphokine release. Howard et al (1983) and Inaba et al (1983) found that release of B cell stimulating lymphokines could be induced from an activated T cell by IL2. Kelso et al (1984) have also reported that IL2 can enhance the rate of MAF release from T cell clones. These results suggest that not all lymphokine release is antigen controlled or that alternative pathways may exist for triggering. For the study of the antigen signal, it is useful to define conditions whereby this signal is the sole requirement for triggering lymphokine release. As a general rule, two necessary and sufficient conditions are: (i) non-cooperation between T cells (which can be simply achieved by showing that the T cell dose response curve is first order) and (ii) the use of target cells incapable of contributing to the reaction.

The behaviour of lymphokine releasing cells in relation to cell dose and time were examined in chapter 7 and were found to be complex. However, the complexity could be explained by assuming that triggering of lymphokine release

was an all or none event. Analysis of antigen dose response curves obtained at different times after antigen exposure also suggested that T cells recycle and require new target cells after about 5 hours. This is probably due to the killing of target cells following T cell interaction. Thus, dose response curves determined within 5 hours of antigen exposure can be interpreted as being simply a function of the number of triggered T cells. Two immediate applications of this conclusion are suggested. First, in a heterogeneous population of T cells it is possible to calculate the proportion of cells reactive to different target cells by directly comparing the titre obtained in target cell excess. For example, the linear section of figures 6.3 and 6.4 are produced in target cell excess and can be interpreted as showing that 100-fold more T cells are triggered by Con A than by $NaIO_4$. Conversely a cloned T cell population could be used to detect the number of antigenic cells in separate heterogeneous populations of target cells. They could also be used to examine the parameters which control the effectiveness of target cell-T cell interaction, i.e. why do some T cells require two target cells and others one before lymphokine triggering is started?

A number of steps can be defined which would allow the application of these quantitative methods to the detection of any lymphokine releasing T cell. These include:

1) Choice of a lymphokine whose activity is relevant to the reaction of interest,
2) Choice of triggering conditions whereby lymphokine release is solely antigen dependent,
3) Use of assay conditions whereby the background release of lymphokine is negligible. For in vivo generated cells this may require a short in vitro culture (For example if cells were triggered to release lymphokine just prior to cell preparation, then incubation of the cells for a few hours at a high dilution, to prevent further cell interaction, would reduce this release).

An assay with these characteristics would then be subject to the rules of quantitation established with experimental model systems. Such an assay could therefore be used to estimate activated T cell numbers, measure antigen specificity and also the antigen triggering 'efficacy' of different target cells.

The importance of CsA as an immunological tool

Lymphokine release was used to study the immunosuppressive properties of the drug CsA. This work found that CsA acts selectively and reversibly on the lymphokine release reaction at a site after antigen binding and before transcription of lymphokine encoding mRNA. CsA does not affect other T cell activities such as cytotoxicity and IL2 dependent proliferation. This specific site of action offers a wide range of potential uses in dissecting the role

of the antigen signal in immune reactions. Using this drug the antigen signal was shown to be required continuously to maintain lymphokine release from the cell. When added at any time after antigen stimulation, the rate of lymphokine release remained steady for two hours and then rapidly decreased. This finding suggests that the lymphokine release machinery must continually receive antigen signals in order to to operate.

CsA is non-toxic for cells and appears to selectively act on lymphocytes. These properties allow its use *in vivo* to examine signal-dependent T cell reactions. One such study was described in chapter 5. The finding that acute rejection of pancreatic islet grafts by lyt 2^+ T cells is lymphokine dependent, is perhaps surprising and emphasises the importance of analysing reactions *in vivo* rather than simply assuming a function from *in vitro* analysis. Identifying which particular lymphokines are important for this reaction can be approached using the technique of incorporating T cell clones along with the graft (in the manner seven day-activated T cells were used in chapter 5). As clones release a distinct repertoire of lymphokines, different cells could be selected for the release of different activities and tested for there ability to reject tissue allografts. Other T cell dependent reactions which could be analysed using this drug include: the role of the antigen signal in maintaining T cell memory and the relative contribution of lymphokine release (CsA sensitive) and

cognate signalling (which is unlikely to be CsA sensitive) in T-B cell collaboration in immunoglobulin class switching.

The T cell antigen signal

The lymphocyte antigen signal must be triggered before an adaptive immune response can be generated. Clearly the triggering characteristics of this signal will play an important role in the development of immunity. In this thesis a number of new methods were employed to explore the T cell antigen signal.

An antigen signalling mechanism is comprised of at least two separate reaction steps. These can be represented by receptor binding and by the ability of the bound antigen to trigger the signal. A suggested term for the property of the antigen which allows it to confer the second reaction is the intrinsic activity (section 1.12). The importance of the intrinsic activity in T cell receptor triggering was shown by experiments described in chapter 6 where it was found that the T cell mitogenic lectin Con A will not trigger the antigen signal unless bound to another cell. This occured despite the observation of Chilson *et al* (1984) that this mitogen will bind to the T cell receptor.

In chapter 7 the conclusion was reached that the antigen signal is all or none, while in chapter 4 CsA treatment revealed that the signal must be transferred continuously to

maintain a cell in the lymphokine release 'mode'. In summary, the findings which relate to the characteristics of the T cell antigen signal are:

1) the signal is all or none,
2) triggering depends on the physical properties of the antigen, and
3) the signal must be continuously maintained to trigger the release of a fixed set of lymphokine activities.

Such a fixed behaviour pattern is reminiscent of B lymphocytes which will only produce one immunoglobulin isotype at any time. Together these observations suggest a modification to the clonal selection theory which would make a provision for describing and predicting the development of a particular class of immune response. The original rule "one cell, one specificity" (Burnet, 1957) could be changed to read:

> One cell, one specificity, one triggering mechanism and one pattern of effector activity.

According to this rule immune response heterogeneity would be set by the prior programming status of the lymphocytes involved. For T lymphocytes this heterogeneity will be determined by the lymphokine repertoire. In discussing the application of the cellular paradigm to the problem of response heterogeneity (chapter 1) the importance of 3 mechanisms for the generation of lymphocyte diversity were described. It is interesting to reiterate them here

using the T cell lymphokine repertoire as the specific example of lymphocyte heterogeneity. Writing the T cell activation reaction in the manner of Lafferty et al (1983) yields;

$$T_{(x)} + S_x \xrightarrow{1}{2} T'_{(x)} \xrightarrow{1} \ell$$

where $T_{(x)}$ is an antigen reactive T cell, S_x is the antigen bearing S^+ cell, T' is the activated T cell, 1 is the antigen signal, 2 represents the second or costimulation signal and ℓ represents the lymphokine repertoire. It can be seen that a different T cell response could be selected by antigen if clonally distributed T cells existed which possessed different triggering mechanisms (and consequently perceived intrinsic differences between antigen classes). The resulting activated T cell population could then express different "ℓ". Alternatively the selected T cell could express a different response because a history of prior activation events has led to an altered program (assuming that activated T cells return to the resting state). Another area of potential heterogeneity would be the nature of the second signal. Although IL1 is the best known costimulator there is no evidence to suggest it is the only one. There are a number of cell types which can act as S^+ cells such as splenic dendritic cells (Witmer and Steinman, 1978; Steinman et al, 1983) epidermal Langerhans cells (Stingl et al, 1978) and macrophages from different sites (Minami et al, 1980;

Sunshine et al, 1982). Information regarding the site of antigen exposure could be transferred to the T cell through tissue specific accessory cell costimulators. This mechanism could also pattern responses to different antigen classes. Assuming that manifold accessory cells possess different antigen processing capabilities (depending, for example, on the physical properties of the antigen or on associated opsonins) the information could be transferred to the T cell by accessory cell-specific costimulators. According to this view, T cells express a number of different potential 'second signal receptors' following antigen exposure. Costimulation through different receptors ultimately affects the lymphokine repertoire of the activated T cell. Another means by which information about site of antigen exposure could be mediated is via environmental or site-specific hormones and/or by resident cell types capable of initiating non-antigen signals to the cell. Either mechanism could alter the T cell program.

In the introduction to this thesis it was observed that different response patterns probably evolved to counter different classes of infectious disease or antigen challenge. Although undoubtedly useful to the survival of the species, the heterogeneity in response is a problem for the design of new vaccines. For example, a vaccine may present viral antigen in a form which stimulates an immune response more appropriate for eliminating a soluble protein than fighting a viral infection. The ability to control the

class of immune response is an important component of the "central problem" assigned to immunologists (see section 1.1). One step towards the solution of any problem is to define it in a solvable form and to develop procedures suitable for its analysis. The lymphokine release assay, described in the previous section, should prove useful for examining the lymphokine repertoire generated by T cells from different sites and following stimulation with different S^+ cells or costimulators. This assay also has the ability to detect the intrinsic activity requirements for antigen signalling of T cells activated following a particular priming protocol. A projected technical problem is that T cells isolated from peripheral lymphoid organs represent a diverse collection of different programs due to their different histories of stimulation. To counter this problem it would be necessary to work with 'virgin' lymphocytes. Possible sources of these cells would be the thymus or foetal lymphoid tissue.

A THEORETICAL APPENDIX

This appendix is included in support of a deductive method of describing immune phenomena and to illustrate how quantitative arguments can be found to explain so called helper effects in _in vitro_ T cell culture.

A note on method

The immune response in some sense can be viewed as a semi-autonomous organism. The different receptor and programmed response properties of its cellular components confer on this 'organism' a number of potential behaviour patterns. The stochastic nature of cellular interaction _in vivo_ and the influence of the structured lymphoid organs on the ability of cells to interact and signal one another will probably ensure that no accurate _quantitative_ theory will soon be provided which could be used to predict the speed and level of an immune response. However, the observed response of isolated immune cell types to various signals can be used as the foundation for the application of a deductive scientific method to predict the _qualitative_ outcome of an immune response. Evidence for this assertion is the work of Lafferty, _et al_ (1983) who showed that from a small number of fundamental empirical observations a number of properties of graft rejection can be predicted. A further example is supplied by Bevan and Matzinger (1977) who showed that the empirically derived rules of T cell specificity can be adapted to predict the very high proportion of cells reactive to foreign MHC.

Ideally this scientific method of deducing complex behaviour from the fundamental properties of the constitutive cells could be formalized. The consistent application of this method would yield a deductive theory (expressed in terms of elements and rules governing their behaviour) which would (hopefully) define the parameters involved in stimulation of all classes of immune response.

When applying this method, if a deduced behaviour is found not to correspond to the observed in vivo behaviour, one of two problems would be indicated. Either, some premise of the theory is incorrect or an element not accounted for in the theory is exerting some influence. Unfortunately for cellular immunology, the latter explanation is usually chosen and takes the form of a new cell type with just the right properties to balance the phenomenon with the original theory (for example, a suppressor T cell). Any science has as its domain, elements for description. The complexity of any theory developed within that domain will be an exponential function of the number of elements it seeks to describe. As cells are a prominent part of an immunologists work, new ones should only be postulated when all other avenues of explanation are exhausted. Another problem with assuming new elements for description is the increasing difficulty for any theory to be predictive. When considering a theory to predict the qualitative behaviour of an immune response it is not necessary to admit supposed cell types which modulate or alter the quantitative outcome of a

reaction. In fact, when considering lymphocytes it should not be necessary to assume many more lymphocytes than there are classes of adaptive immune reaction.

Steps toward a deductive theory of immunology

The Lafferty theory depends upon two postulates. The first is a statement of a number of experimental characteristics of T cell activation. That is: two signals antigen and costimulator (CoS), are required for initiation of T cell activation. This is expressed:

$$T \xrightarrow[2]{1} T'$$

where T is the resting T cell, T' the activated T cell and 1 and 2 represent the antigen and costimulation signals respectively. A corollary of this postulate is that a cell capable of providing CoS (an S^+ cell) is required for T cell activation.

The second postulate is that a control structure on the S^+ cell surface regulates the release of CoS activity. Although there is no direct evidence for this postulate, it can be seen as a reasonable assumption based on a number of experimental findings. They are; 1) that S^+ cells do not constitutively produce CoS, 2) that CoS is found in the supernatant following interaction of T cells with antigenic S^+ cells and 3) that some mitogens can stimulate the release of CoS from S^+ cells.

From these two simple rules Lafferty et al (1983) show that the control stucture of CoS release will behave precisely like the MHC of the species.

This theory however, makes no provision for a distinction between the activation of class 1 and class 2 restricted T cells. Thus, it cannot explain the findings of Ertl (1981) regarding the development of class 1 restricted T cell reponses to viruses (described in section, 1.8). When the empirical conclusion derived from the work with the response to different viruses is included in the formulation of Lafferty a theory results which is more generally applicable than that of Lafferty.

The conclusion to be drawn from examination of T cell responses to viruses is that a class 1 MHC-restricted T cell response cannot be generated unless the antigen has some mechanism for incorporation into a cell membrane. The sentiment expressed in this experimental observation will be referred to as the "law of class distinction".

The requirement for an S^+ cell to present the antigen to effect T cell activation allows the law of class distinction and the Lafferty theory to be combined in the following way.

Postulate A: Antigens which form part of the S^+ cell surface will generate class 1 MHC restricted responses following interaction with a receptive T cell repertoire. i.e.

$$ {}^{y}S + \tau \xrightarrow{1} T'_{(Ky)} $$

where y represents the antigen while its position - superscripted in front of the S cell - indicates that it is an integral part of the cell surface, τ indicates the T cell population. The subscripted brackets represent the T cell receptor and the enclosed 2-letter expression gives the specificity. In this case "K" represents the class 1 MHC antigens. This process will be referred to as <u>passive presentation</u> of antigen.

Postulate B: Antigens which cannot become part of the cell membrane must be actively processed by the S^+ cell. Antigens presented in this way will stimulate class 2 restricted T cell responses. The processing step can be expressed:

$$ S + y \longrightarrow S_y, $$

and the reaction referred to as active processing. The distinction between active and passive antigens is given by the position of the antigen symbol

The reaction with T cells is written:

$$ S_y + \tau \longrightarrow T'_{(Iy)} $$

where I represents the class 2 MHC antigens.

These reactions encapsulate the law of class distinction into the Lafferty theory. Most of the predictions made by Lafferty which relate to alloreactivity and the rejection of grafts still apply although the modification does differ in some details regarding grafts which differ at minor histocompatibiltiy antigens. These differences are currently being tested and will not be discussed here. From the discussion so far a number of conclusions can be deduced.

One implication of these reactions is that to produce a class 1 restricted T cell response to a virus then a stimulating cell must be infected (unless it is enveloped and can fuse with the S^+ cell membrane). Viral antigen products produced in other cell types or introduced as a killed vaccine or purified protein product must be processed and hence will generate a class 2 MHC restricted T cell response but no class 1 response. As class 1 MHC antigens are expressed on all cells then the response restricted by this antigen is likely to be the most effective in eliminating viruses. This has in fact been demonstrated for immunity to influenza virus (Leung and Ada, 1982). Clearly, if a virus were to always kill or compromise in some way the S^+ cell, then a class 1 resticted response would be impossible. In this case class 2 restricted responses would occur as these do not require the cell to be infected. The immune response to the herpes group of viruses displays these characteristics. Class 1 MHC restricted T cells are very difficult to demonstrate following infection with these

viruses despite the fact that virus specific antibody is detected (Pfizenmaier et al, 1977). Whether this characteristic is due to the mechanism provided above is amenable to experiment.

The theory also encompasses alloreactivity following a similar argument to that used by Matzinger and Bevan (1977). The T cell specificity repertoire is normally randomly expressed and tolerant of self antigens. This tolerance must include all 'processed' self products (assuming for the moment that processing is indiscriminate) as well as antigenic components of the cell membrane. The 'rules' of T cell specificity (deduced from this theory because MHC must be involved in antigen recognition before the T cell can receive the second activation signal) can be stated;

The interaction antigen (My) $\neq$ (M_1y) i.e. Restriction

The interaction antigen (My) $\neq$ (My_1) i.e. Specificity

M represents the restricting MHC allele and y represents the antigen. The "1" subscript distinguishes different allelic forms.

Considering the case of passive presentation of antigen by S^+ cells, an alteration in K, the class 1 MHC antigen, will mean that all normal and self cell surface products

will by rule 1 above (restriction), appear foreign to the T cell repertoire. It is not known how many different non-MHC antigens appear on a cell surface but it is probably over one hundred. This figure represents the proportional increase of new antigens created by changing a single MHC allele compared to the incorporation of a new foreign antigen into the cell surface membrane. This reaction can be expressed as:

$$y_S K^1 + y_\tau K \frac{1}{2} \underset{(K^1 y)}{y_T, K}$$

In these reactions the K and K^1 superscripts are used to distinguish cells carrying separate class one MHC alleles. The symbol y in this reaction represents all cell surface molecules and is therefore made up of individual molecules y_1, y_2, y_3 ... y_n, where n is the number of different cell surface molecules. Clearly, incorporation of a single new molecule such as might occur following a virus infection, will provide 'n'-fold less new antigens than will the alteration of the class 1 MHC antigen.

A similar argument can be used to explain Class 2 MHC alloreactivity. In the case where a new class 2 antigen is included in the stimulating cell then all processed molecules, self or not, will appear foreign. Once again the number of reactive T cells will be a function of the number of molecules potentially processed by the S^+ cell. This number will again be the factor by which a class 2 MHC

allogeneic S^+ cell will be stimulatory over the processing of one foreign antigen. These conclusions depend on the assumption that antigen processing cells process indiscriminately any protein. It is difficult to imagine how alternative options such as selective processing only of foreign antigen could be mediated by a phagocyte without the power of discrimation borne by the entire lymphocyte repertoire. As the question of how antigen presenting cells select antigen for processing is still an open one, the above theory of class 2 alloreactivity remains.

Thus, by this theory, alloreactivity can be shown to be a direct consequence of T cell stimulation by two classes of antigen. This theory also explains why allogeneic cells reactive to class 1 MHC antigens express the lyt 2 antigen typical of class 1-MHC restricted T cells (by this theory they are class 1 MHC-restricted T cells) whereas class 2 MHC alloreactive T cells display the cell surface phenotype of T cells restricted by the same antigen.

Precisely the same mechanism can be invoked to explain the large number of T cells responsive to oxidized (Novogrodsky and Katchalski 1971) or haptenated cells (Shearer 1974). Chemical treatment of the MHC would be (immunologically) equivalent to a foreign MHC allele, and induce both class 1 and class 2 'alloreactions'. This prediction is borne out by the finding in chapter 6 that $NaIO_4$ does not act directly on the T cell but that cells

must interact to detect the alterations on the cell surface. The fact that the alloreactive T cells used for analysis of mitogen behaviour recognize oxidized cells could be due to cross-reactivity of a number of interaction antigens. That is, foreign MHC and some minor histocompatibility antigen would cross-react with oxidized MHC and a minor antigen (not necessarily the same antigen). This redundancy in allospecificity runs counter to the observation that cytotoxic T cells are sensitive to small antigenic changes. However, the large number of antigens predicted for cells carrying altered MHC provides a much greater chance of cross reactivity for a given restricted T cell with a particular alloantigen. Hunig and Bevan (1982) have demonstrated that a T cell clone reactive to H-2^d plus a BALB/c minor antigen will also recognize a cell carrying H-2^k and a different BALB/c background antigen.

The method by which lectins fit into this scheme has already been discussed (section 6.4).

Quality versus quantity in interpeting data

The addition of two more empirical rules leads to a number of important observations relating to lymphocyte behaviour in vitro. These two rules are:

That antigen alone triggers lymphokine release (including IL2). i.e.

$$T' \xrightarrow{1} IL2$$

That activated T cells proliferate in response to IL2. i.e.

$$T' + IL2 \longrightarrow nT'$$

The set of reactions we have do not allow any quantitative statements to be made of the reaction <u>in vivo</u>. However, when the cells are isolated and devoid of structure they will behave in a fashion dependent on the interactive behaviour of the individual components.

The model predicts a number of reactions which will take place in an MLR. For instance in an MLR between strains differing at a class 1 MHC allele only, the following reactions apply:

(1) Release of CoS:

$$y_S{}^K + y_{T^{K1}_{(Ky)}} \longrightarrow CoS$$

(2) Antigen signalling of T cells:

$$y_p{}^K + y_\tau{}^{K1} \longrightarrow T^{1/2}_{(Ky)}$$

where p is an antigen bearing cell (not necessarily an S^+ cell-type but including them) and $T^{1/2}$ is an antigen triggered T cell before costimulation.

(3) Activation:

$$T^{1/2}_{(Ky)} + CoS \longrightarrow T'_{(Ky)}$$

(4) IL2 release:

$$T'_{(Ky)} + {}^{y}p^{K} \longrightarrow IL2$$

(5) Proliferation:

$$T'_{(Ky)} + IL2 \longrightarrow nT'$$

(6) reactions 4 and 5 repeated.

I have no formal model of this reaction as many of the putative steps have no experimentally defined dose response function, however, it is interesting to observe the behaviour of this reaction assuming the simplest possible model, that is, all antigen requirements are fulfilled for each step and that linear relationships apply at each dose response. In this case, consider the distinction between a minor histocompatibility antigen versus a major antigen disparity between the two cultured cell types. This theory has already shown how many more cells will be reactive to the major antigen. Taking the conservative estimate that 10-fold less T cells recognize the minor antigen leads to the following deduction.

Reaction 1. Release of CoS; 10-fold less T cells will trigger the release of 10-fold less CoS.

Reaction 2. Antigen signalling. I have assumed all antigen components are fulfilled therefore, 10 fold less $T^{1/2}$ will form in response to the minor antigen.

Reaction 3. Activation. In the culture with the minor difference 1/10 the CoS concentration with 1/10 the T cell number yields 1/100 the number of T' compared to the culture with the major difference.

Reaction 4. IL2 release. Again antigen requirements are fulfilled, therefore the difference in the IL2 concentration between the two cultures will be one hundred fold.

Reaction 5. Proliferation 1/100 the [IL2] plus 1/100 the number of T' generates 1/10000 the number of T' in the minor culture compared to the major.

Already this is a massive difference, if we took another IL2 release and proliferation step into account the figure would become; 1/10 000 the T' plus 1/10 000 the IL2 concentration gives $1/10^8$ difference in the number of T'. All this results from a 10-fold difference in starting concentration of reactive T cells and assuming the simple linear relation at each step and perfect antigen stimualtion. Quite obviously the actual functions are not straight lines, however the point is made that the number of reactions involved multiply differences profoundly at each step. This quantitative argument can account for the observation that reactions to minor angtigens and other antigens are much weaker <u>in vitro</u> than those which occur in a MLR. The above argument holds equally for active and passive antigens. Although when both occur in the culture it is interesting to note the possible cooperative effects which can occur due to

the fact that no distinction has been made concerning the action of the soluble mediators IL2 and CoS.

In an MLR for instance between cells bearing both class 1 and class 2 differences adopting a protocol similar to that described by Cantor and Boyse (1975) treating with anti ly 1 and C' (assuming this is a marker for class 2 MHC reactive T cells) effectively halves the number of reactive cells in culture. Therefore when assaying cytotoxic cell generation (assuming the targets are only Class 1 MHC positive) produces a 2^5-fold decrease in T' number or a 1/32 reduction in the number of 'cytotoxic' cells. Such a dramatic effect would conventionally be used to argue for the existence of a helper cell type obligatory for generation of cytotoxic cells. Clearly, however, assuming no more than 2 classes of T cell with different phenotype can explain very large in vitro helper effects without assuming any qualitative change to the behaviour of the constituent cell population*.

The complexity of these in vitro reactions is, in fact, almost certainly greater than I have indicated here. Consider for example reaction 3, IL2 release. This is the reaction studied in chapters 3 and 7 of this thesis. In

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* It is interesting to note that in the paper by Cantor and Boyse which showed T-T cooperation the authors make no claim for obligatory help. They found that treatment with anti-ly 1 and C' reduced the cytotoxic response only if they did not reconstitute viable cell numbers. Reconstitution following antisera treatment resulted in a 2-fold increase in the number of cytotoxic T cells. The concept of obligatory T-T help appears to have come later with the erroneous concept that only ly 1^+ T cells can produce IL2.

chapter 7 it was demonstrated that, depending on the T cell number and the antigen cell dose, an increase in T cell number could produce more, less, or the same amount of IL2. This occurs because of the requirement for the binding of 2 target cells. Incorporating this into the above argument, it is clear that the effect of changing any cell number in an <u>in vitro</u> T cell culture can have profound influences - either positive or negative. Most importantly, changes in the outcome of a reaction due to changes to cell populations are not necessarily due to qualitative changes in behaviour of the interacting cell types. The conclusion? Either <u>in vitro</u> T cell behaviour should be carefully modelled by studying it's component parts in order to anticipate possible quantitative effects, or, the reaction should not be used at all to study cell interactions.

Conclusions

The above discussion illustrates how a large number of diverse immune phenomena, not obviously related, can be deduced from simple rules of T cell behaviour. The reactions used;

"Control structure" $S_x^K + T_{(Kx)} \rightarrow CoS$

"Costimulation" $T \xrightarrow{1}_{2} T'$

"Class distinction" $S_x + \rightarrow T'_{(Ix)}$

$^{y}S + \rightarrow T'_{(Ky)}$

"Lymphokine release" $T' \xrightarrow{1} \ell$

"Proliferation" $T' + IL2 \rightarrow nT'$

all have an empirical basis. Some of the reactions assumed are poorly understood and certainly need to be more adequately demonstrated under a number of different test conditions. The success of the deduced conclusions from these rules perhaps serve as an illustration of how advances could be made by studying the fundamental units of cell behaviour and not the myriad possible permutations of their higher level interaction.

CHAPTER 9

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